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(57) Abstract

Mammalian synovial phospholipase A_2 ($sPLA_2$) enzymes are provided, as well as DNA constructs encoding these enzymes, methods of producing the enzymes recombinantly, and antibodies thereto. Therapeutic methods employing anti-synovial phospholipase antibodies are also provided, in addition to diagnostic methods and other applications of $sPLA_2$.

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SYNOVIAL PHOSPHOLIPASES

Related Application

This application is a continuation-in-part of Serial No. 215,726, filed 6 July 1988, which is a continuation-in-part of Serial No. 089,883, filed 27 August 1987, the disclosures of which are incorporated by reference herein.

Technical Field

The present invention relates to the isolation, characterization, and production by recombinant means of proteins. More particularly, the present invention is related to synovial phospholipase A_2.

Background

Inflammatory disorders presently account for a significant percentage of debilitating diseases. Chronic conditions, such as rheumatoid arthritis, systemic lupus, psoriasis, and possibly atherosclerosis, stem from inflammatory reactions in the joints, skin and blood vessels. It is now apparent that a central role in the inflammatory reaction is the production of phospholipid metabolites called eicosanoids. It is generally accepted that in most tissues the synthesis of the eicosanoids is limited by the availability of arachidonic acid (AA) which is liberated from esterified

stores in complex lipids. The liberation of AA is accomplished by the activity of phospholipases.

Phospholipase A_2 (PLA_2; EC3.1.1.4) catalyzes the release of fatty acids from the sn^2 position of 1,2-diacyl-sn-glycero-3-phosphocholines. The best characterized varieties are the digestive enzymes secreted as zymogens in the pancreas of mammals. Amino acid sequences and cDNAs have been cloned for pancreatic PLA_2 enzymes from a variety of mammals. See, e.g., O'Hara et al. (1976) J Biochem 99:733-739; Dufton et al. (1983) Eur J Biochem 137:537-544; Grataroli et al. (1982) Eur J Biochem 122:111-117. These mammalian PLA_2 enzymes have a close homology to venom phospholipases of snakes and bees. Dufton et al., supra. In particular, the key active site residues and the alignment of cysteines appear to be highly conserved. X-ray crystallographic studies of bovine pancreatic PLA_2, along with several venom enzymes, have led to the development of detailed models for PLA_2 enzyme structure and mechanism of action. See, e.g., Renetseder et al. (1985) J Biol Chem 260:11627-11634. Both pancreatic and venom PLA_2 have been shown to be proinflammatory. Pruzanski et al. (1986) J Invest Dermatol 86:380-383. An additional digestive PLA_2 has been isolated from pig intestine and a partial amino acid sequence deduced. Verger et al. (1982) Biochemistry 21:6883-6889.

The structure of pancreatic PLA_2 has been used as a model for designing novel PLA_2 inhibitors. This approach, however, has not led to the design of a drug which has proved effective in inhibiting inflammation in vivo.

If PLA_2 plays a central role in mammalian inflammatory disease, however, it probably is not through any of the digestive forms in most instances. Rather, analogous PLA_2 enzymes, referred to as "cellular" PLA_2 enzymes appear to be the likely regulator of AA release during the onset of inflammation. Unfortunately, these cellular PLA_2 enzymes are not well understood. This is due to the fact that they are difficult to obtain in quantity and require more extensive purification than the digestive forms of PLA_2.

Cellular forms of PLA_2 have been isolated from a wide variety of mammalian tissues and cell types, including brain (Gray & Strickland, 1982, Can J Biochim 60:108-117), liver (DeWinter et al., 1982, Biochim Biophys Acta 712:332-341), lung (Franson et al. ,1982, Lung 160:275-284; Garcia et al., 1975, Biochim Biophys Res Comm 64:128-135; Sahu & Lynn, 1977, Biochim Biophys Acta 489:307-317), intestine (Verger et al., 1982, Biochemistry 21:6883-6889), spleen (Teramoto et al., 1983, J Biochim 93:1353-1360), macrophages (Trotter & Smith, 1986, Neurochem Res 11:349-361; Lanni & Franson, 1981, Biochim Biophys Acta 658:54-63; Vadas & Hay, 1980, Life Sciences 26:1721-1729; Vadas et al., 1981, Nature 293:583; Wightman et al., 1981, Biochim J 200:441-444; Franson et al., 1973, Biochim Biophys Acta 296:365-373), leukocytes Traynor & Authi, 1981, Biochim Biophys Acta 665:571-577; Franson et al., 1977, Biochim J 167:839-841), erythrocytes (Kramer et al., 1978, Biochim Biophys Acta 507:381-394), ascitic fluid (Forst et al., 1986, Biochemistry 25:8381-8385), chondrocytes (Chang et al., 1986, J Immunol 136:1283-1287), and platelets (Hayakawa et al., 1988 J Biochem 103:263-266; Hayakawa

et al., 1987, J Biochim 101:1311-1314; Jesse & Franson, 1979, Biochim Biophys Acta 575:467-470; Apitz-Castro et al., 1979, Biochim Biophys Res Comm 91:1, 63-71). For a review, see Van Den Bosch (1980) Biochim Biophys Acta 604:191-246. See also, commonly owned U.S. Patent Application, Serial No. 946,557, filed 24 December 1986.

Of particular interest is the isolation of a PLA_2 from inflammatory exudates, such as the synovial fluid of rheumatoid arthritis patients. Stefanski et al., (1986) J Biochim 100:1297-1303; Vadas et al. (1985) Life Sciences 36:579-587; Vadas & Pruzanski (1984) Adv Inflammation Res 7:51-59; Vadas et al. (1981) Nature 293:583-585; Pruzanski et al. (1985) J Rheumatol 12:211-216; Silverman et al., American Rheumatism Ass'n: 51st Annual Scientific Meeting (9-13 June 1987, Washington, D.C.); Pruzanski et al., ibid.

Of these various cellular enzymes, the reports of their activity differ in size, pH optima, substrate specificity, Ca^{++} requirement, form (soluble vs. membrane-associated), and abundance. Since no complete protein sequences have been publicly reported for these isolates (partial sequences published by Verger et al., 1982, supra; Forst et al., 1986, supra; Hayakawa et al., 1987, supra; and Hayakawa et al., 1988 supra), it is difficult to say which, if any, of these isolates represent the same enzymes. Moreover, it is difficult to completely discriminate between PLA_1 and PLA_2 directly in all but highly purified isolates, since cleavage at the sn^2 position of phospholipids can also be the result from the combined sequential activities of PLA_1 and lysophospholipase. As can be seen, however, many of these enzymes have been prepared from cells associated

with inflammatory responses (i.e., macrophages, leukocytes, chondrocytes, synoviocytes, etc.) or inflammatory exudates. Nevertheless, the lack of cause/effect data has made it difficult to establish which, if any, of these enzymes are key in the inflammatory response.

The isolation of the PLA_2 form responsible for rheumatoid arthritis in vivo would provide an important tool useful in the design of anti-inflammatory drugs. Based on the work with digestive and venom PLA_2 inhibitors, it is believed that the form(s) of PLA_2 responsible for inflammatory disease, while similar, are sufficiently different in structure such that inhibitors of digestive or venom PLA_2 do not necessarily inhibit the latter form in vivo. Thus, to efficiently design specific inhibitors, it is necessary to isolate the specific PLA_2(s) that are involved in rheumatoid arthritis in sufficient quantity so that it can be structurally characterized. PLA_2 is also generally useful in the food processing industry (Dutilh & Groger, 1981, J Sci Food Agric 32:451-458) and the preservation of fish. Mazeaud & Bilinski (1976) J Fish Res Board Can 33:1297-1302.

Summary of the Invention

According to the present invention, it has been discovered that a new family of mammalian phospholipase A_2, hereinafter referred to as synovial phospholipases A_2 (synovial PLA_2 or $sPLA_2$), are encoded within the mammalian genome, and are substantially different from the known PLA_2 enzymes in both DNA and amino acid sequences. The cloning of the genes for $sPLA_2$ provides for the structural characterization of these new

enzymes, as well as methods of producing them in substantial and purified quantities. Thus, the present invention provides, inter alia, an important tool useful in the design of anti-inflammatory drugs.

In one embodiment, the present invention provides a composition containing double-stranded DNA construct comprising a heterologous region, said region comprising a coding sequence for a mammalian synovial phospholipase A_2, said composition being substantially free of constructs that do not contain said heterologous region. This DNA construct may or may not be contained within a replicon.

In another embodiment, the present invention provides a method of producing a recombinant mammalian synovial phospholipase A_2 comprising: providing a population of transformed cells comprising a replicon functional in said cells, said replicon comprising a coding sequence under the control of a promoter functional in said cells, said coding sequence encoding a mammalian synovial phospholipase A_2, said population being substantially free of other cells; growing said population under conditions whereby said mammalian synovial phospholipase A_2 is expressed; and recovering said mammalian synovial phospholipase A_2. The method of the present invention can employ any suitable procaryotic or eucaryotic expression system.

In a further embodiment, the present invention provides a composition comprising mammalian synovial phospholipase A_2 substantially free of contaminating proteins.

In still another embodiment, the present invention provides anti-mammalian synovial phospholipase

A_2 antibody, and methods of treating inflammatory disorders employing anti-mammalian synovial phospholipase A_2 antibodies.

Description of the Figures

Figure 1 shows a comparison between the N-terminal amino acid sequences of synovial phospholipases of the present invention and other phospholipases. hRASF-Peak A and Peak-B are two synovial PLA_2s isolated from human synovial fluid. NP is the "non-pancreatic" type of PLA_2 described in copending U.S. Patent Application, Serial No. 946,557, including the human (h), porcine (p) and rat (r) forms. The sequence designated "h cln 10" is derived from clone λSPLA2-10 (Figure 4) and may be an $sPLA_2$ type B or C sequence or a different PLA_2. Also shown in the figure are several pancreatic PLA_2s: porcine intestinal PLA_2 (p Intestine), rabbit ascites PLA_2 (rab Ascites), rat platelet PLA_2 (r platelet); and two snake venoms: Crotalus atrox (C. atrox), and Agkistrodon piscivorus (A. pisc K-49).

Figure 2 is a C_4 reverse phase HPLC profile of partially purified synovial PLA_2 showing the enzyme activity and optical density profile.

Figure 3 shows the DNA sequence of two 50-mer oligonucleotide probes used to identify synovial PLA_2 clones.

Figure 4 shows the DNA sequences of two human PLA_2 genomic clones, λSPLA2-6, and λSPLA2-10, which contain exons of the two PLA_2 enzymes described herein.

Figure 5 shows a 60-mer oligonucleotide probe synthesized to match amino acid residues 5-24 of $sPLA_2$

type A shown in Figure 1 and based on the nucleotide sequence of clone λsPLA2-6.

Figure 6 shows the nucleotide sequence and deduced amino acid sequence from a cDNA clone for human $sPLA_2$ type A, designated λSPLA2cDNA-4.

Figure 7 shows the nucleotide sequence of exons 1-5 from genomic clone λSPLA2-6 of human $sPLA_2$ type A.

Figure 8 shows oligonucleotide linkers useful in recombinant DNA constructs for the expression of $sPLA_2$ in E. coli.

Figure 9 graphically depicts the accumulation of PLA_2 enzyme activity in serum-free medium during infection of CV-1 cells by recombinant vaccinia virus containing the human $sPLA_2$ type A gene.

Detailed Description

The practice of the present invention will employ, unless otherwise indicated, conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual" (1982); "DNA Cloning: a Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. 1985); "Transcription And Translation" (B.D. Hames & S.J. Higgins eds. 1984); "Animal Cell Culture" (R.I. Freshney ed. 1986); "Immobilized Cells And Enzymes" (IRL Press, 1986); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

In describing the present invention, the following terminology will be used in accordance with the definitions set out below.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication in vivo; i.e., capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its normal, double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3'

(carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the translation start codon of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Procaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

A coding sequence is "under the control" of the promoter sequence in a cell when RNA polymerase which binds the promoter sequence transcribes the coding sequence into mRNA which is then in turn translated into the protein encoded by the coding sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell wall. Exogenous DNA may or may not be integrated

(covalently linked) to chromosomal DNA making up the genome of the cell. In procaryotes and yeast, for example, the exogenous DNA may be maintained on an episomal element such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

Two DNA sequences are "substantially homologous" when at least about 85% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides match over the defined length of the DNA sequences. Sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., supra; DNA Cloning, Vols. I & II, supra; Nucleic Acid Hybridization, supra.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian

genomic DNA in the genome of the source organism. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., a cDNA where the genomic coding sequence contains introns, or synthetic sequences having codons different than the native gene). Allelic variations or naturally occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

A protein composition is "substantially free of contaminating proteins" when at least about 75% by weight of the protein in the composition is the particular protein of interest. Preferably, this protein comprises at least about 90% by weight of the protein in the composition, most preferably at least about 99% by weight. It is also preferred that a protein composition, which is substantially free of contaminating proteins, contain only a single molecular weight species having the activity of the protein of interest.

"Synovial phospholipase A_2" (synovial PLA_2 or $sPLA_2$) refers to the class of mammalian enzymes exhibiting PLA_2 activity and found in the synovial fluid of a mammal (such as a human) afflicted with rheumatoid arthritis. It is believed that $sPLA_2$ enzymes are produced by inflamed synovial tissue, or perhaps granulocytes or macrophages in the synovial fluid. Synovial PLA_2 enzymes are characterized in having a molecular weight of about 15 ± 3 kD when measured by polyacrylamide gel electrophoresis (PAGE) (12.5% polyacrylamide gel, 0.1% SDS). Representative of the family of enzyme are $sPLA_2$ type A, type B and type C. The NH_2-terminal amino acid sequence of types A and B

are shown in Figure 1. The complete amino acid sequence of type A, deduced from the human cDNA clone λPLA$_2$cDNA-4, is shown in Figure 6. Type A is present in synovial fluid from all types of arthritis examined. Type B varies in abundance from complete absence in some rheumatoid samples to about 33% of the total activity in other samples. Type B typically appears at higher levels in fluid samples from osteoarthritis patients than in samples from rheumatoid patient, but type A still constitutes the majority of sPLA$_2$. Type B also shows considerable stimulation in hydrolytic activity relative to type A in the presence of either 0.5 M Tris or 0.1% Na deoxycholate; type A is inhibited by 0.5 M Tris. Type C, when present, is two- to five-fold less abundant than type B. These extracellular enzymes are (i) soluble, (ii) calcium-dependent, (iii) have proinflammatory activity in tissue when injected intradermally or intraarticularly, and (iv) exhibit absolute specificity for the sn-2 acylester bond of dipalmitoylphosphatidylcholine. This characterization also includes synthetic and recombinant analogs of sPLA$_2$ wherein any resulting changes, deletions or additions in the amino acid sequence does not change the above characteristic activities.

The sequences compared in Figure 1 show that sPLA$_2$ resembles other PLA$_2$ sequences in the number and placement of the 14 Cys residues, particularly the "type II" enzymes, of which C. atrox PLA$_2$ is an example. Synovial PLA$_2$ also lacks a Cys at position 11, which is characteristic of the highly pro-inflammatory type II enzymes (e.g., Viperid snake venom forms, and PLA$_2$ species described in copending U.S. Serial No. 946,557).

The comparison demonstrates that $sPLA_2$ is distinct from all other known PLA_2 sequences, particularly in the variable regions near the carboxy terminus. A twenty residue prepeptide, containing a typical signal for translocation across a cellular membrane is present upstream of the mature enzyme sequence, and is presumably cleaved during or after synthesis.

A clone, λSPLA2-6, of genomic DNA encoding $sPLA_2$ type A has been deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville, MD, U.S.A. 20852, on 14 August 1987, and given accession no. 40361. The coding sequence (Figure 4) is on a 404 bp AluI fragment which can be isolated from λSPLA2-6. An additional clone, which may represent a human genomic sequence from at least one exon of $sPLA_2$ type B or C, and called λSPLA2-10, was also deposited on 14 August 1987 with the ATCC under accession no. 40360. The coding sequence in this clone is contained on an AluI fragment of about 460 bp. A cDNA clone encoding all of human $sPLA_2$ type A on an 854 bp EcoRI fragment, designated λSPLA2cDNA-4, was deposited with the ATCC on 27 May 1988 under accession no. 40456. An expression vector containing an $sPLA_2$ coding sequence, p86-1A (discussed below), was also deposited with the ATCC on June 27, 1988 under accession no. 67735. These deposits will be maintained under the terms of the Budapest Treaty. The $sPLA_2$ coding sequences of λSPLA2-6, λSPLA2-10 and λSPLA2cDNA-4, and the expression cassette sequence of pHNF86 are incorporated herein by reference. In the event of any discrepancy between a sequence disclosed herein and the sequence of a deposited clone, the clone's sequence is controlling.

While it is possible to purify $sPLA_2$ from an appropriate tissue/fluid source (see below), it is preferred to produce it by recombinant methods. A DNA sequence encoding $sPLA_2$ can be isolated by one of several approaches. These methods will rely in part on nucleic acid hybridization using appropriate oligonucleotide probes. Such probes can be constructed synthetically based on the $sPLA_2$ DNA or amino acid sequences disclosed herein, or isolated from the genomic $sPLA_2$ clones also described herein.

The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., DNA Cloning: VOL. I (D.P. Glover ed. 1985); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds. 1985); Oligonucleotide Synthesis (M.J. Gate ed. 1984); T. Maniatis et al., Molecular Cloning:a Laboratory Manual (1982); B. Perbal, A Practical Guide To Molecular Cloning (1984). First, a DNA library is prepared. The library can consist of a genomic DNA library from a selected mammal, such as a human. Human genomic libraries are known in the art. See, e.g., Maniatis et al. (1978) Cell 15:687-701; Lawn et al. (1978) Cell 15:1157-1174. DNA libraries can also be constructed of cDNA prepared from poly-A RNA (mRNA) by reverse transcription. See, e.g., U.S. Patent Nos. 4,446,325; 4,440,859; 4,433,140; 4,431,7400; 4,370,417; 4,363,877. The mRNA is isolated from a cell line or tissue believed to express $sPLA_2$, such as synovial tissue or inflammatory cells isolated from synovial fluid . The preferred source of mRNA for cDNA library constructions is

synovial joint tissue. The genomic DNA or cDNA is cloned into a vector suitable for construction of a library. A preferred vector is a bacteriophage vector, such as any of the phage lambda. The construction of an appropriate library is within the skill of the art. See, e.g., B. Perbal, supra.

Once the library is constructed, oligonucleotides are used to probe the library to identify the segment carrying the $sPLA_2$ coding sequence. In general, the probes are preferably based upon known nucleic acid sequences. However, if the later is unknown, it may be desirable to base probes upon an amino acid sequence determined from a purified $sPLA_2$. In the latter case, nucleotide sequences are selected so as to correspond to the codons encoding the amino acid sequence. Since the genetic code is redundant, it will usually be necessary to synthesize several oligonucleotides to cover all, or a reasonable number, of the possible nucleotide sequences which encode a particular region of the protein. Thus, it is generally preferred in selecting a region upon which to base the probes, that the region not contain amino acids whose codons are highly degenerate. It may not be necessary, however, to prepare probes containing codons whose usage is rare in the mammal from which the library was prepared.

In certain circumstances, one of skill in the art may find it desirable to prepare probes that are fairly long and/or encompass regions of the amino acid sequence which would have a high degree of redundancy in the corresponding nucleic acid sequences. Probes covering the complete gene, or a substantial part of the gene, may also be appropriate, depending upon the

expected degree of homology. Due to the highly conserved nature of PLA_2 across species lines, it is likely that full length sPLA2cDNA probes from one species, such as the human clone λSPLA2cDNA-4, can be readily used to screen libraries prepared from another species. In other cases, it may be desirable to use two sets of probes simultaneously, each to a different region of the gene. While the exact length of any probe employed is not critical, generally it is recognized in the art that probes from about 14 to about 20 base pairs are usually effective. Longer probes of about 25 to about 60 base pairs are also used.

As is known in the art, oligonucleotide probes are labeled with a marker, such as a radionucleotide or biotin, using standard procedures. The labeled set of probes is then used in the screening step, which consists of allowing the single-stranded probe to hybridize to isolated ssDNA from the library, according to standard techniques. Either stringent or permissive hybridization conditions could be appropriate, depending upon several factors including, but not limited to, the length of the probe, whether the probe and library are from the same species, and whether the species are evolutionarily close or distant. It is within the skill of the art to optimize hybridization conditions so that homologous sequences are isolated and detectable above background hybridizations. The basic requirement is that hybridization conditions be of sufficient stringency so that selective hybridization occurs; i.e., hybridization is due to a minimum degree of nucleic acid homology (e.g., at least about 75%), as opposed to non-specific binding or hybridization due to a lower degree

of homology. See generally, "Nucleic Acid Hybridization," supra. Once a clone from the screened library has been identified by positive hybridization, it can be further characterized by restriction enzyme analysis and DNA sequencing to confirm that the particular clone contains a coding sequence for $sPLA_2$.

Partial genomic clones, such as the clone of an exon of $sPLA_2$ in λSPLA2-10, can be extended into complete clones by one of several techniques. A clone can be extended in either the 5' or 3' direction using "chromosome walking" techniques to ensure inclusion of the entire gene coding region. Restriction fragments of these clones can then be probed with, for example, $sPLA_2$ cDNA. If sufficient homology exists within these exons to pancreatic PLA_2, other exons of $sPLA_2$ could be identified with pancreatic $sPLA_2$ clone, also. When using non-$sPLA_2$ cDNA probes, it is particularly preferred to probe with oligonucleotides which correspond to particularly conserved regions (e.g., amino acid residues 44-52), which would allow prediction of possible differences (e.g., Asp_{49} changed to Lys_{49}).

Other coding regions in genomic clones may be rapidly identified by direct sequencing of the DNA downstream of a cloned exon using modern M13-dideoxy sequencing techniques. The sequence is then inspected in all three reading frames to reveal an open reading frame. Other exons will also be apparent since they will be bounded on both sides by intron-splicing signals and should encode conserved amino acids.

More specifically, now that the correct gene coding sequence for an exon of $sPLA_2$ type B or C is known, it can be used to obtain the entire protein

coding region of the enzyme by one or more of the following means. First, the exon can be trimmed from the λ clone and placed in a more convenient vector, such as pBR322, so that large quantities of DNA containing only the exon itself can be obtained and used as a hybridization probe. Alternately, a 60-mer oligonucleotide corresponding to the unique regions of the coding region (e.g., amino acid residues 6-25) can be synthesized. Either can be used as a hybridization probe for northern blots of mRNA obtained from various sources, such as, peritoneal cells and pus, endothelial tissue, and peripheral blood leukocytes, lymphocytes, and macrophages. In addition, mRNA from various cell lines such as differentiated U-937 and HL60 can also be tested. Any tissue or cell source containing detectable levels of hybridizing mRNA is then used to produce a cDNA library which will then be screened with the same probes in order to detect a full-length cDNA encoding $sPLA_2$. Indeed, as described below, this strategy lead to the cloning of full length cDNA's encoding sPLA type A, such as clone λSPLA2cDNA-4.

Alternately, in the absence of a good tissue source for the mRNA, it may become necessary to obtain internal sequences from the type B or C protein. This can be done, for example, by Staph-V8 proteolysis of peak A material purified in the usual way (described below), followed by reductive alkylation and separation by HPLC of the digestion products. Elution peaks corresponding to discrete enzyme fragments can then be sequenced as before. Alternatively, putative amino acid sequences from cDNA clones can be employed. From the resulting sequence, oligonucleotides can be designed and

produced for use as hybridization probes to locate the other exons. Ultimately, the isolated exons are ligated together in such a way that the correct mature protein is encoded.

Mammalian genomic clones (partial or full-length) containing the longest inserts of the $sPLA_2$ gene can be co-transfected into Chinese hamster ovary (CHO) cells with plasmid DNA containing a marker, such as neomycin and metallothionine resistance genes. Surviving cells selected in the presence of antibiotic G418 and Cd^{++}, and surviving clones can be analyzed for the presence of $sPLA_2$-hybridizing transcripts in a Northern blot of extracted RNA. Clones containing the desired transcripts can then be used as an mRNA source for a cDNA library construction.

Synovial PLA_2 can be purified from human synovial fluid from patients afflicted with rheumatoid arthritis or psoriasis. The purification protocols, described in detail below, allow for the first time the purification of native $sPLA_2$ in sufficient quantity and at a high enough purity to permit accurate amino acid sequencing. The amino acid sequences derived from the purified $sPLA_2$'s allow for the design of probes to aid in the isolation of native $sPLA_2$ nucleic acid sequence, or the design of synthetic nucleic acid sequences encoding the amino acid sequence of a $sPLA_2$.

Specific anti-sera or monoclonal antibodies (described below) can be made to a synthetic $sPLA_2$ peptide having the sequence of amino acid residues, such as those shown at the NH_2-terminus in Figure 1. Particularly preferred is a peptide spanning positions 1 through 26. This is a unique region of the protein, and

antibodies thereto can be used to immunoprecipitate any $sPLA_2$ present in a selected tissue, cell extract, or body fluid. Purified $sPLA_2$ from this source can then be sequenced and used as a basis for designing specific probes as described above. Antibodies to other regions that diverge from known PLA_2s can also be used. Also useful as antigens are purified native or recombinant $sPLA_2$.

As mentioned above, a DNA sequence encoding $sPLA_2$ can be prepared synthetically rather than cloned. The DNA sequence can be designed with the appropriate codons for the $sPLA_2$ amino acid sequence. In general, one will select preferred codons for the intended host if the sequence will be used for expression. The complete sequence is assembled from overlapping oligonucleotides prepared by standard methods and assembled into a complete coding sequence. See, e.g., Edge (1981) Nature 292:756; Nambair et al. (1984) Science 223:1299; Jay et al. (1984) J Biol Chem 259:6311.

Synthetic DNA sequences allow convenient construction of genes which will express $sPLA_2$ analogs or "muteins". Alternatively, DNA encoding muteins can be made by site-directed mutagenesis of native $sPLA_2$ genes or cDNAs, and muteins can be made directly using conventional polypeptide synthesis. Of particular interest in the construction of muteins is changing the catalytic His_{48} residue in type A to another amino acid, such as Gln. Position 48 muteins may act as a PLA_2 inhibitor by binding to endogenous inflammatory PLA_2 enzymes, thereby creating inactive dimers. Other potential targets for mutagenic alteration include the three basic residues near the N-terminus (positions 7, 10 and 16), which may

be involved in interaction with membrane-associated substrates. Muteins altered in any one or all of these positions by the substitution of acidic residues (e.g., Glu or Asp) could have reduced activity toward membrane-bound or complex substrates.

Site-directed mutagenesis is conducted using a primer synthetic oligonucleotide complementary to a single stranded phage DNA to be mutagenized except for limited mismatching, representing the desired mutation. Briefly, the synthetic oligonucleotide is used as a primer to direct synthesis of a strand complementary to the phage, and the resulting double-stranded DNA is transformed into a phage-supporting host bacterium. Cultures of the transformed bacteria are plated in top agar, permitting plaque formation from single cells which harbor the phage.

Theoretically, 50% of the new plaques will contain the phage having, as a single strand, the mutated form; 50% will have the original sequence. The resulting plaques are hybridized with kinased synthetic primer at a temperature which permits hybridization of an exact match, but at which the mismatches with the original strand are sufficient to prevent hybridization. Plaques which hybridize with the probe are then picked, cultured, and the DNA recovered.

Once a coding sequence for $sPLA_2$ has been prepared or isolated, it can be cloned into any suitable vector or replicon and thereby maintained in a composition which is substantially free of vectors that do not contain an $sPLA_2$ coding sequence (e.g., free of other library clones). Numerous cloning vectors are known to those of skill in the art, and the selection of an

appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the various bacteriophage lambda vectors (E. coli), pBR322 (E. coli), pACYC177 (E. coli), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria), pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), actinophage, ϕC31 (Streptomyces), YIp5 (Saccharomyces), YCp19 (Saccharomyces), and bovine papilloma virus (mammalian cells). See generally, DNA Cloning: Vols. I & II, supra; T. Maniatis et al., supra; B. Perbal, supra.

According to the present invention, the coding sequence for mammalian $sPLA_2$ is placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding $sPLA_2$ is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. If the coding sequence contains a signal peptide, it may or may not be the $sPLA_2$ signal sequence. In bacteria for example, mature $sPLA_2$ is preferably made by the expression of a coding sequence which does not contain the $sPLA_2$ signal peptide, or by expression of a coding sequence containing a leader sequence which is removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

An expression vector is constructed according to the present invention so that the $sPLA_2$ coding

sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). The control sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site. For expression of $sPLA_2$ in procaryotes and yeast, the control sequences will necessarily be heterologous to the coding sequence. If the host cell is a procaryote, it is also necessary that the coding sequence be free of introns (e.g., cDNA). If the selected host cell is a mammalian cell, the control sequences can be heterologous or homologous to the $sPLA_2$ coding sequence, and the coding sequence can either be genomic DNA containing introns or cDNA. Either genomic or cDNA coding sequences can be expressed in yeast.

A number of procaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Pub. Nos. GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Pub. No. 103,395. Preferred procaryotic expression systems are in E. coli. Other preferred expression vectors are those for use in eucaryotic systems. See, e.g., commonly owned U.S.

Patent Application Serial No. 809,163, filed 16 December 1985, the disclosure of which is incorporated herein. A preferred eucaryotic expression system is that employing vaccinia virus, which is well-known in the art. See, e.g., Mackett et al. (1984) J Virol 49:857; "DNA Cloning," Vol. II, pp. 191-211, supra; PCT Pub. No. WO 86/07593. Yeast expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Pub. Nos. 103,409; 100,561; 96,491. Another preferred expression system is vector pHS1, which transforms Chinese hamster ovary cells. The use of the vector is described in PCT Pub. No. WO 87/02062 and commonly owned U.S. Patent Application Serial No. 804,692, filed 4 December 1985, the disclosure of which is incorporated herein by reference.

Depending on the expression system and host selected, $sPLA_2$ is produced by growing host cells transformed by an expression vector described above under conditions whereby the $sPLA_2$ protein is expressed. The enzyme protein is then isolated from the host cells and purified. If the expression system secretes the enzyme into growth media, the protein can be purified directly from cell-free media. If the $sPLA_2$ protein is not secreted, it is isolated from cell lysates. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

Native, recombinant or synthetic $sPLA_2$ peptides (full length or subunits) can be used to produce both polyclonal and monoclonal antibodies. If polyclonal antibodies are desired, purified $sPLA_2$ peptide is used to immunize a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) and serum from the

immunized animal later collected and treated according to known procedures. Compositions containing polyclonal antibodies to a variety of antigens in addition to $sPLA_2$ can be made substantially free of antibodies which are not anti-$sPLA_2$ by immunoaffinity chromatography.

Monoclonal anti-$sPLA_2$ antibodies can also be readily produced by one skilled in the art from the disclosure herein. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., "Hybridoma Techniques" (1980); Hammerling et al., "Monoclonal Antibodies And T-cell Hybridomas" (1981); Kennett et al., "Monoclonal Antibodies" (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,451,570; 4,466,917; 4,472,500; 4,491,632; 4,493,890.

Panels of monoclonal antibodies produced against $sPLA_2$ peptides can be screened for various properties; i.e., isotype, epitope, affinity, etc. Of particular interest are monoclonal antibodies that neutralize the activity of $sPLA_2$. Such monoclonals can be readily identified in PLA_2 activity assays. High affinity antibodies are also useful in immunoaffinity purification of native or recombinant $sPLA_2$.

The discovery of pancreatic PLA_2 expressed in human lung tissue indicates that the pancreatic form may play a larger role than had been expected in inflammatory disease. Thus, antibodies to any other PLA_2 forms described herein (both polyclonal and monoclonal) can be

used to treat inflammatory disorders. Anti-pancreatic PLA_2 antibody can be produced as described herein for anti-$sPLA_2$ antibody. If the disease is acute endotoxic shock, for example, the appropriate therapeutic method would be to treat the patient with an effective dose of anti-PLA_2 antibodies (e.g., anti-synovial PLA_2) through a conventional intravenous route. In the treatment of local, acute inflammation, treatment with anti-$sPLA_2$ antibody would be indicated, perhaps by intramuscular injection. It is particularly preferred to treat local, chronic inflammation, such as joints of rheumatoid arthritis patients, by parenteral administration of anti-$sPLA_2$ antibody. These compositions may also be useful in treating other forms of arthritis, such as osteoarthritis. Since endotoxic shock induces elevated levels of PLA_2, it may also be desirable to administer anti-PLA_2 antibodies in conjunction with other therapies directed to the gram-negative pathogens and their toxins (e.g., anti-LPS therapy). Since PLA_2 is also known to attack the pulmonary surfactant monolayer, in the case of respiratory distress (e.g., adult respiratory distress syndrome) it may be desirable to administer anti-PLA_2 antibodies by inhalation combined with replacement pulmonary surfactant phospholipid, dipalmitoyl phosphatidylcholine. PLA_2 antagonists, such as $sPLA_2$ muteins, could also be used in place of antibodies.

The determination of the appropriate treatment regimen (i.e., dosage, frequency of administration, systemic vs. local, etc.) is within the skill of the art. For administration, the antibodies will be formulated in a unit dosage injectable form (solution, suspension,

emulsion, etc.) in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are usually nontoxic and nontherapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and Hank's solution. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. A preferred vehicle is 5% (w/w) human albumin in saline. The vehicle may contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability, e.g., buffers and preservatives. The antibody is typically formulated in such vehicles at concentrations of about 1 µg/ml to 10 mg/ml.

Anti-sPLA$_2$ antibodies will also be useful in diagnostic applications. For example, synovial fluid isolated from rheumatoid arthritis patients shows that it contains primarily, if not completely, PLA$_2$ of the type A variety. On the other hand, samples from osteoarthritis patients typically contain appreciable amounts of type B as well as type A, usually in a 2:1 ratio of type A to type B based upon activity in the presence of 50 mM Tris. Thus, the present invention contemplates a method, particularly a diagnostic method, in which a synovial fluid sample from a human (or other mammal) is provided, and the amounts of sPLA$_2$ type A and type B are quantitatively measured in an assay and compared. For example, employing anti-sPLA$_2$ antibodies specific to type A or to type B in a quantitative immunoassay could be used to distinguish between the two types of arthritis. Antibody specific for type A or type B could be formulated into any conventional immunoassay format; e.g., homogeneous or heterogeneous, radioimmunoassay or ELISA. The various formats are well

known to those skilled in the art. See, e.g., "Immunoassay: A Practical Guide" (D.W. Chan and M.T. Perlstein eds. 1987) the disclosure of which is incorporated herein by reference. Quantitative assays other than immunoassays could also be used to measure the relative levels of type A and type B $sPLA_2$.

In general, recombinant production of $sPLA_2$ can provide compositions of that enzyme substantially free of contaminating proteins. The ability to obtain high levels of purity is a result of recombinant expression systems which can produce $sPLA_2$ in substantial quantities vis-a-vis in vivo sources. Thus, by applying conventional techniques to recombinant cultures, $sPLA_2$ compositions can be produced that are substantially more pure than the cellular PLA_2 compositions presently available from non-digestive and non-venom sources.

The purified $sPLA_2$ compositions of the present invention are useful in several regards. First, they can be used in food processing technology as described in Dutilh & Greger (1981) J Sci Food Agric 32:451-458. In addition, $sPLA_2$ compositions can be used to delay the onset of rancidity in fish. See, e.g., Mazeaud & Bilinski (1976) J Fish Res Board Can 333:1297-1302.

Purified $sPLA_2$, however, will be particularly useful as a tool in the design and screening of inflammation inhibitors. First, milligram amounts of the material are obtainable according to the present invention. Milligram amounts are capable of crystallization to permit three dimensional studies using X-ray diffraction and computer analysis. This may permit deduction concerning the shape of the molecule, thus defining proper shapes for substances useable as inhibitors of

the enzyme activity normally exhibited by $sPLA_2$. Inhibitors have already been designed for "converting enzyme", the catalyst for the subsequent conversion of angiotensin I into angiotensin II. Generally, these antagonists have been "dipeptides" whose interactions with converting enzyme are stabilized by modification of the "residues" participating in the peptide bond so as to enhance the ability of the "dipeptide" to interact specifically with converting enzyme. Thus the peptide bond joins specifically chosen carboxylic acids and amines (not necessarily amino acids). These "dipeptides" are configured in a three dimensional array so as to complement the contours of the intended target, converting enzyme. A similar lock and key spatial arrangement may result from molecules designed complementary to the surface contours of the crystallized $sPLA_2$ of the invention. It is understood that "surface" includes convolutions which may face inward, and specifically includes the active site. Furthermore, "complementary" is understood to mean that, in addition to spatial conformations which "fit", interactions between the protein and the molecule which matches its surface contours are attractive and positive. These interactions may be hydrogen bonding, ionic, or hydrophobic affinity.

Accordingly, the invention contemplates peptide antagonists (2-15 amino acids) to $sPLA_2$ which are characterized by three dimensional contours complementary to the three dimensional contours on the surface of recombinant $sPLA_2$. By peptide in this context is meant that the antagonist contains carboxylic acid amide bonds corresponding to one less than the number of

residues. The carboxylic acid and amine participants need not be α-amino acids.

Second, even without the assistance of a three dimensional structure determination, purified $sPLA_2$ of the invention is of significance as a reagent in screening $sPLA_2$ inhibitors in vitro as an ad hoc approach to evaluation. Impure $sPLA_2$ preparations currently available yield confusing data due to the impact of the impurities on the test results. For example, contaminants which turn out to be themselves inhibitors, activators, or substrates for $sPLA_2$ will interfere with the evaluation. Thus, a substantial improvement in current screening techniques for $sPLA_2$ inhibitors would be effected by the availability of the purified human $sPLA_2$ protein.

The $sPLA_2$ compositions described herein may also be useful as an anti-cancer drug. For example, direct injection of $sPLA_2$ into, or in the vicinity of malignant tumors, and optionally in conjunction with tumor excision, will result in high levels of powerful chemoattractants for, and activators of, macrophages. These activated macrophages may then enhance localized tumor reduction or elimination.

Still another application of purified $sPLA_2$ according to the present invention is as an adjuvant in a vaccine composition. The formulation of vaccines is well known in the art. Usually, vaccine formulations include the antigen(s) (e.g., attenuated virus, killed virus, viral polypeptide subunits, killed bacteria, bacterial pili, etc.) in a pharmaceutically acceptable parenteral vehicle. The improved vaccine composition of the present invention may contain, in addition to an

$sPLA_2$ adjuvant, an additional adjuvant. The concentration of $sPLA_2$ in the final vaccine formulations can be readily determined by one of ordinary skill in the art. Typically, but not always, the concentration of $sPLA_2$ will be from about 1 ng/ml to about 1 µg/ml.

Described below are examples of the present invention which are provided only for illustrative purposes. They are not intended to limit the scope of the present invention in any way as numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art in light of the present disclosure. Those of ordinary skill in the art are presumed to be familiar (or to have ready access to) the references cited in the application, and the disclosures thereof are incorporated by reference herein.

Examples

I. Purification and sequencing of $sPLA_2$

A. Initial purification

Sephadex® G-75, CM-Sephadex® C-50, and protein standards for gel filtration and electrophoresis were purchased from Pharmacia Fine Chemicals. Acrylamide, N,N,N',N'-tetramethylethylenediamine, bromophenol blue, Coomassie brilliant blue R, sodium dodecyl sulfate (SDS), fatty acid free bovine serum albumin (BSA), dipalmitoylphosphatidylcholine, and Lowry protein assay kit were obtained from Sigma. Silver stain and Bio-Rad protein assay kit were purchased from Bio-Rad Laboratories.

1-[^{14}C]Oleic acid (50 mCi/mmol) was purchased from New England Nuclear. 2-[1-^{14}C]-palmitoyl-1-palmitoylphosphatidylcholine (59 mCi/mmol) and 2-[1-^{14}C]-linoleoylphosphatidylethanolamine was supplied by Avanti Polar Lipids (Birmingham, AL). Ampholine PAG plate, pH 3.5-9.5, for analytical electrofocusing was purchased from LKB Bromma. Precoated thin layer chromatography (TLC) silica gel 60 plates were obtained from BDH. All chemicals and reagents used were of analytical grade.

Synovial fluids (SF) were obtained from patients with active classical or definite rheumatoid arthritis (RA) by arthrocentesis. This material was centrifuged at 4°C to remove cells and debris, pooled and stored in polypropylene tubes at -70°C until required.

All purification procedures were carried at at 4°C. Pooled synovial fluid (510 ml) was dialyzed against 5 mM buffer, pH 5.0, for 24 h. The resultant precipitate was redissolved in 0.5 M acetate buffer, pH 5.00, and applied to a 200 ml column of CM Sephadex® C50 which had been equilibrated with the same buffer. The column was sequentially eluted with 0.5 M acetate buffer, pH 5.0; 0.3 M NaCl in 0.2 M Tris-HCl, pH 8.5; and 3 M NaCl in 0.2 M Tris HCl, pH 8.5. The PLA_2 was eluted in the latter buffer. Fractions containing PLA_2 activity were pooled, dialyzed against 0.05 M Tris-HCl, pH 8.5, and lyophilized. The lyophilized residue was reconstituted in 0.05 M Tris-HCl buffer, pH 8.5, continuing 2 M NaCl and chromatographed on a 1.6 x 68 cm Sephadex® G75 column, which had been equilibrated with the same buffer.

The column was eluted at 20 ml/h and fractions of 2.8 ml were collected for determination of PLA_2 activity and protein content. Active fractions were pooled, dialyzed against 0.05 M Tris-HCl, pH 8.5, and lyophilized. The residue was dissolved in 0.0625 M Tris-HCl, pH 9.5, containing 1% SDS and 10% glycerol, incubated for 1 h at 37°C and applied to a 15% polyacrylamide gel. Preparative electrophoresis was carried out at 30 mA for 4 h. The gel was cut into 0.5 cm strips. The protein was crushed and eluted with 0.1 M Tris-HCl buffer, pH 7.5. Fractions containing PLA_2 activity were pooled and lyophilized. The steps of purification and enrichment are summarized in Table 1.

Table 1

Purification step	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min·mg protein)	Purification	Yield (%)
Synovial fluid	21,930	18,717	0.85	1	100
Dialysis	6,528	13,702	2.09	2.5	73
CM-Sephadex® C-50	6.6	3,388	513.33	603.9	18
Sephadex® G-75	1.0	2,046	2,046.00	2,407.1	11
Preparative SDS-PAGE	0.195	758	3,887.18	4,573.2	4

Polyacrylamide gel electrophoresis (PAGE) in 15% polyacrylamide gels was performed in the presence of 0.1% SDS as described by Laemmli (1970) Nature 227:680-681. Ovalbumin, carbonic anhydrase, trypsin inhibitor, and lactalbumin were used as molecular weight markers. The samples were incubated in 0.0625 M Tris-HCl, pH 6.8, containing 2% SDS and 10% glycerol with 5% 2-mercaptoethanol (2-ME) for 6 min at 100°C for analytical PAGE or without 2-ME for 1 h at 37°C for preparative SDS-PAGE, and then applied to the gel. Electrophoresis was performed for 4 h at 30 mA and the protein bands were stained with Coomassie brilliant blue or Bio-Rad silver stain. Switzer et al. (1975) Anal Biochim 98:231-237.

Polyacrylamide gel electrophoresis of the post G-75 fraction (1.5 mcg) in the presence of sodium dodecyl sulfate and 2-mercaptoethanol indicated the presence of two protein bands corresponding to molecular weights of 17 K and 15 K. An identical electrophoretic pattern was obtained for the same preparation of PLA_2, without reduction of disulfate bonds. PLA_2 activity was associated with both the 15K and 17K bands.

Protein determinations for all PLA_2 preparations described in Examples I.A. or II, except eluates from SDS-PAGE, were performed by the Bio-Rad method. Bradford (1976) Anal Biochim 72:248-254. The protein eluted from SDS-PAGE was assayed by the method of Lowry following trichloroacetic acid precipitation. Peterson (1977) Anal Biochim 83:346-356. Bovine serum albumin served as a protein standard for both methods.

B. Final purification

The material from the initial purification was loaded onto a reverse-phase C-4 HPLC column and eluted with a 15-60% acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The eluted fractions were assayed for PLA_2 activity (C, below), and the active fractions were pooled and lyophilized overnight in a siliconized Falcon #2059 tube. The peaks of activity, termed peaks A, B and C, were obtained routinely and were further purified separately (Figure 2). The lyophilized peak material was resuspended in a PAGE loading buffer (2.3% SDS, 50 mM Tris, 10% glycerol), heated at 90°C for 3 min, and loaded onto a 12.5% acrylamide minigel. Then 40,000 dpm of ^{125}I-labeled porcine propancreatic PLA_2 was included within the sample as an autoradiographic marker. After electrophoresis, the gel was autoradiographed for 30 min, and the gel was cut into 1.0 mm slices, using the autoradiogram as a cutting guide. The slices were crushed and the activity was eluted in 10 mM N-ethylmorpholino acetate, pH 7.0, for 1-2 days. Assays were performed on 1.0 µl of the eluate after 60 min incubation at 37°C, and an activity profile was obtained (Figure 2). Peaks A, B and C all eluted from the slices corresponding to 15,000 MW, just ahead of the propancreatic marker. Active fractions were spotted and dried directly onto quaternary amine glass fiber filter paper. The filters then were washed four times in the same buffer, 5 min each, 38and dried. Sequence analyses were performed via Edman degradation on an Applied Biosystems gas phase sequencer. The NH_2-terminal

sequences of $sPLA_2$ type A (peak A) and $sPLA_2$ type B (peak B) are shown in Figure 1.

C. Phospholipase A_2 assays

Standard assay conditions for final purification steps consisted of 50 mM Tris, pH 8.0, 150 mM NaCl, 5.0 mM $CaCl_2$, 0.04% sodium deoxycholate (DOC), and 0.22 nmoles of 1-stearoyl-2-[1-^{14}C]arachidonyl-L-3-phosphatidylcholine (PC, Amersham #CFA.655) as substrate, incubated at 37°C for 30 min. The substrate was prepared by dissolving freshly desiccated PC in 2% DOC, which was then diluted to the appropriate concentration in assay buffer. The 50 µl reaction was started by the addition of prewarmed substrate and terminated by the addition of 10 µl 8 M acetic acid. Fifty microliters of the reaction mixture was spotted and dried onto Whatman thin-layer chromatography plates, and the plates were chromatographed using chloroform: methanol:acetic acid (90:10:1) as a solvent. The dried plates were exposed overnight with X-ray film, or alternatively the bands corresponding to product (arachidonate) and substrate (PC) were scraped and counted in scintillation fluid.

II. Characterization of Synovial PLA2

The material prepared in Example I.A. was further characterized as described below.

A. pH Dependence and Substrate Specificity

Phospholipase activity was quantitated by the modified method [Vadas et al. (1980) Life Sci 26:1721-1729] of Franson et al. (1978) J Lipid Res 19:18-23,

using autoclaved Escherichia coli, strain $K_{12}C_{600}$, labeled with [^{14}C]oleic acid, as the substrate. Assays were performed in substrate excess, using 2.8 x 10^8 E. coli per assay, corresponding to 5.6 nmol of phospholipid with a specific activity of 4,120 cpm/nmol phospholipid. The standard reaction mixture of 1.5 ml total volume contained 10 mg BSA, 7 mM $CaCl_2$, 0.1 M Tris-HCl buffer, and [^{14}C]oleate-labeled E. coli. Reactions were allowed to proceed for 30 min at 37°C and were terminated by filtration through a 0.45 µm Millipore filter. Enzyme activities were corrected for non-enzymatic hydrolysis. Under conditions of substrate excess, the rate of substrate hydrolysis is linear with reaction times of up to 30 min, over a fivefold range of enzymic concentration.

Determination of phospholipase activities towards the radiolabeled synthetic substrates, dipalmitoylphosphatidylcholine, and 2-linoleoyl-1-palmitoylphosphatidylethanolamine, was carried out as described by Shakir, (1981) Anal Biochim 114:64-70. Standard incubation mixtures contained 750 nmol of phospholipid, 2 mM $CaCl_2$, 2 mM sodium deoxycholate (DOC), 0.09% Triton X-100, and enzyme protein in 0.1 M Tris-HCl buffer in a total volume of 400 µl. Incubations were carried out at optimal pH (see below) for 1 h at 37°C in a shaking water bath. The reaction was stopped by addition of 2.0 ml of n-heptane-isopropanol-1 N sulfuric acid (1:4:0.1, v/v/v). Released fatty acids were extracted by the method of Shakir, (1981) Anal Biochim 114:64-70. PLA_2 activity is expressed as nmol of released fatty acid per mg of protein per h.

The pH dependence of purified PLA_2 was determined against dipalmitoylphosphatidylcholine and 1-palmitoyl-2-linoleoylphosphatidylethanolamine over a range of pH 5-10, using the assay of Shakir, (1981) Anal Biochim 114:64-70. Buffers used were of constant ionic strength: 0.1 M sodium acetate-acetic acid (pH 5-6), 0.1 M Tris-HCl (pH 7-8), and 0.1 M glycine-NaOH (pH 9-10).

The pH dependence of purified PLA_2 was studied over the range of pH 5-10 using two synthetic phospholipid substrates, dipalmitoylphosphatidylcholine, and 2-linoleoyl-1-palmitoylphosphatidylethanolamine. Phosphatidylethanolamine was hydrolyzed over a wide range of pH (6-10), with optimal PLA_2 activity evident at pH 7.5-8.0. Maximal PLA_2 activity for phosphatidylcholine was seen at pH 7.0 with an abrupt decrease in activity at pH 8-10.

The specific activities of PLA_2 were comparable for all three phospholipids tested. Membrane phospholipids of E. coli were hydrolyzed most actively, while phosphatidylethanolamine and phosphatidylcholine were hydrolyzed at rates of 41 and 27% respectively of that of E. coli phospholipid. Since detergents (especially the non-ionic Triton X-100) act as an inert matrix solubilizing the phospholipids in similar structures, the observed activities of phosphatidylethanolamine and phosphatidylcholine are directly comparable. Roberts et al. (1978) J Biol Chem 253:1252-1257.

Table II

Phospholipid substrate	Activity (nmol/μ protein.h)
E. coli phospholipid	122.5
Dipalmitoylphosphatidylcholine	33.5
1-Palmitoyl-2-linoleoyl-phosphatidylethanolamine	49.9

* E. coli membrane phospholipid composition: 48.6% phosphatidylethanolamine, 25.0% phosphatidylglycerol, and 11.1% cardiolipin. Vadas & Pruzanski, (1984) Adv Inflam Res 7:51-59.

B. Determination of Positional Specificity

The positional specificity of the purified enzyme was determined usin 1-palmitoyl-2-[1-^{14}C]-palmitoylphosphatidylcholine as substrate. The assay system contained 750 nmol of radioactive phosphatidylcholine dispersed in 0.1 M Tris-HCl buffer, pH 7.5, containing 2mM $CaCl_2$, 2mM DOC, 0.09% Triton X-100, and 100 μl of enzyme preparation in a total volume of 400 μl. The reaction was carried out at 37°C for 3.5 h and was terminated by the addition of 8 ml of chloroform-methanol (2:1,v/v). Lipids were extracted by the method of Folch et al., (1957) J Biol Chem 226:497-509, and

separated by TLC in chloroform-methanol-acetic acid-water (65:25:8:4, v/v/v/v). The lipid spots were visualized by exposure to iodine vapor. After sublimination of the iodine, spots corresponding to authentic PC, lyso PC and free fatty acid standards were scraped into scintillation vials containing 10 ml of scintillation fluid and measured for radioactivity in a liquid scintillation spectrometer (Beckman LS7500). 1-acyl-2-[1-^{14}C]palmitoylphosphatidylcholine was incubated with venom PLA_2, crude synovial fluid or purified synovial fluid PLA_2 and the reactions products were analyzed by thin-layer chromatography.

Greater than 93% of the total substrate was hydrolyzed by PLA_2 from Crotalus adamanteus venom. Of the total products formed, 97.2% of the radioactivity was associated with free fatty acid, while only 2.8% of radioactive product comigrated with lysolecithin, consistent with preferential cleavage of the fatty acid esterified in the sn-2 position. Similarly, both crude synovial fluid and the purified preparation of PLA hydrolyzed radiolabeled substrate preferentially at the sn-2 position, yielding greater than 95% ^{14}C-fatty acid and less than 5% 2-[^{14}C]palmitoylphosphatidylcholine.

In order to rule out the combined activities of a PLA_1 and lysophospholipase, crude synovial fluid and purified synovial fluid phospholipase were incubated as above with 1-[1-^{14}C]palmitoylphosphatidylcholine. Thinlayer chromatographic analysis of the products revealed that 99% of the radioactive label remained associated with the lysophospholipid substrate, and only 0.05% of the radioactivity was associated with free fatty acid, indicating the virtual absence of detectable

lysophospholipase activity. These data are consistent with an absolute 2-acyl specificity for synovial fluid phospholipase.

C. Effect of SDS on PLA_2 activity

The effect of SDS on the rate of hydrolysis of phosphatidylcholine by highly purified PLA_2 was studied using Shakir's method, supra. SDS inhibited PLA_2 activity on a concentration-dependent manner. Inhibition of PLA_2 was 94 and 7% of initial enzyme activity at SDS concentrations of 1 mg/ml and 0.1 mg/ml, respectively. In evaluating the utility of preparative SDS-PAGE, recovery of enzyme from the slab gel was consistently between 95-100% of the total enzyme applied to the gel. However, subsequent lyophilization of highly purified PLA_2 resulted in significant losses of activity (approx. 64% loss).

D. Immunoreactivity of PLA_2

Synovial fluid PLA_2 was tested for immunoreactivity against rabbit anti-human pancreatic PLA_2 by radioimmunoassay. Sternby et al. (1984) Biochim Biophys Acta 789:164-169. Ten specimens of unfractionated rheumatoid synovial fluid with PLA_2 activity (using E. coli phospholipid substrate) ranging from 8.7 to 31.0 nmol/ml·min were tested. In all cases, there was no substantial cross-reactivity with antihuman pancreatic PLA_2 nor did PLA_2 quantitation by enzyme assay and RIA correlate ($r = 0.134$). Similarly, the antibody failed to recognize the purified (ex-Sephadex® G75) fraction (Table III). The correlation of RIA and

enzyme assay for porcine pancreatic PLA_2 added to synovial fluid was significant.

Table III

Sample	PLA_2 Enzyme activity (nmol/ml·min)	RIA (µg/l)
Synovial fluid 1	8.67	<0.8
2	30.55	0.8
3	23.00	0.8
4	18.23	0.8
5	11.62	<0.8
6	16.37	0.9
7	16.67	4.3
8	18.08	2.1
9	30.73	2.0
10	31.02	2.3
Synovial fluid plus 0 µg pancreatic PLA_2	38.48	<0.8
1 µg pancreatic PLA_2	119.93	13.0
15 µg pancreatic PLA_2	881.98	51.0
30 µg pancreatic PLA_2	1,422.72	64.0
Synovial fluid PLA_2 ex-Sephadex® G-75	81.82	<0.8

III.. Cloning of Synovial PLA_2 Sequences

A. Genomic Cloning

Two 50-mer codon-preference oligos were designed from the RASF peak A sequence, minimizing ambiguity by (a) centering the oligos upon codon groups with minimal ambiguity and (b) allowing for G:T binding. The oligos, shown in Figure 3, were synthesized on an Applied Biosystems oligonucleotide synthesizer.

The oligos were labeled with γ-^{32}P-ATP and polynucleotide kinase, and then used as a hybridization probe for the EMBL3-human leukocyte genomic library obtained from Clonetech Inc. (Mountain View, CA). Then 10^6 total plaques were placed on twenty 150 mm agar plates containing L-broth, using bacterial strain NM538. The plaques were lifted onto nitrocellulose filters, denatured, baked 2 hours at 80°C in a vacuum oven, and prehybridized 2 hours in prehybridization solution (5X Denhardt's, 20% formamide, 6X SSC, 50 mM $NaPO_4$, 100 µg/ml sheared salmon sperm DNA) at 37°C. Hybridization was overnight at 37°C in prehybridization solution plus 10% dextran sulfate and 2 x 10^6 cpm of labeled probe. The filters were washed twice at 25°C in 1X 0.16 M NaCl, 0.016 M sodium citrate (SSC), 0.1% sodium docecyl sulfate (SDS), and then once in the same solution at 50°C for 1 hour and then exposed to autoradiographic film overnight at -70°C. Later, the same filters were rewashed at 55°C and reexposed.

Two classes of signals were seen, 7 of which hybridized to both probes, and 4 of which hybridized to probe 2779 only. All 11 signals were plaque-purified through three rounds of purification. When phage DNA

was prepared from the clones and analyzed by agarose gel separation of restriction enzyme digests, the number of distinct clones was reduced to 2, henceforth represented by clones 6 and 10. Oligo 2779 hybridized strongly when washed at 55°C to clone 6 and 10; oligo 2780 hybridized weakly to clone 6 under these same conditions.

DNA from the two unique clones was digested with endonucleases HaeIII, RsaI, and AluI. The completed digest was extracted with phenol/chloroform and precipitated with ethanol. The dried pellets were resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA, and 1.0 µl aliquots were ligated to bacteriophage M13mp8, which had been previously digested with SmaI. The transformed E. coli strain JM101 cells were plated onto 150 mm L-agar plates and incubated overnight at 37°C. The resulting M13 recombinant plaques were lifted and the filters hybridized as described above. Plaques coinciding with hybridization signals were picked and used to produce single-stranded M13 DNA templates. Sequencing of the clones was done using the dideoxy/enzymatic method, and the resulting sequences were aligned and analyzed on a VACS 8500 computer (Digital Corp.) using the Intelligenetics programs Seq and Gel (Intellicorp Inc., Mountain View, CA). The resulting clone sequences for exons of the two unique phospholipase clones 6 and 10 are shown in Figure 4. They are contained within a 404 bp AluI fragment (clone 6) and an approximately 460 bp AluI fragment (clone 10). Clones 6 and 10 have been renamed λSPLA2-6 and λSPLA2-10, respectively.

The $sPLA_2$ coding sequence in $\lambda sPLA_2$-6 was originally believed to be exon 2 of the human type A gene type A. The cDNA sequence identified in III.B,

below, was used to identify the remaining exons in the genomic clones. It was found that an unexpected intron existed in the 5'-noncoding region of the gene. Thus, what was originally believed to be exon 2 is actually exon 3. The sequence encoding exon 1 is shown in Figure 7. Bases 1016 through 1038 match bases 8 to 27 of the cDNA clone exactly. Although the precise start of transcription has not been determined, its most likely location is at or shortly upstream of base 1012. A potential "TATA" sequence can be seen at nucleotides 968 through 974, and a putative "CAAT" sequence lies at nucleotides 904 through 909.

B. cDNA Cloning

A 60-mer oligonucleotide probe was synthesized to match the nucleotide sequence for λsPLA2-6 shown in Figure 4 and corresponding to the codons for amino acid residues 5-24 shown in Figure 1. This oligonucleotide probe was used to screen RNA blots from various sources, including cell lines HL60 and U937, human synovial cells, human peritoneal inflammatory exudate cells, human pus cells, porcine jejunum tissue, porcine pancreatic tissue, rat spleen tissue, and rat liver tissue. Significant levels of RNA was detected by hybridization in human peritoneal cells and, to a lesser extent, human synovial cells.

A cDNA library was constructed from polyA+ message from a peritoneal cell RNA prep according to the method of Gubler & Hoffman (1983) Gene 25:263-269. The library was screened with the 60-mer probe, and 17 discrete duplicating signals were obtained after washing the filters in 1X SSC, 0.1% SDS at 60°C. DNA from ten

of the clones was subjected to analysis by PAGE. All of the clones contained inserts of 800 to 1,000 bp. Four of the clones, designated 1, 4, 11 and 14, were selected for subcloning into bacteriophage M13 and subsequent DNA sequence analysis by standard techniques. One of these clones, designated λsPLA$_2$cDNA-4 was determined to encode the entire sPLA$_2$ type A sequence. See Figure 6. The other clones contained the same sequence or varied slightly in length at the 5' end and had different length polyA tails. Otherwise, the clones were identical except for a C to T change at position 277, a silent mutation with respect to the amino acid specified by the codon. A typical translation termination sequence, AATAAA, can be seen beginning at base 116. The mature peptide sequence encoded by λPLA$_2$cDNA-4 contains 124 amino acids, and has a calculated molecular weight of 13,919 daltons.

IV. Recombinant Synovial PLA$_2$

A. Bacterial Hosts

Active recombinant sPLA$_2$ was produced in bacteria, such as E. coli, as a β-galactosidase fusion protein employing a procedure adapted from de Geus et al. (1987) Nucleic Acids Res 15:3743-3759. This methodology was adapted to sPLA$_2$ as follows. First, a single base change from C to G introduced at the C-terminus in nucleotide 588 created a HindIII site 17 bases downstream from the TGA stop codon. This change was made via oligonucleotide-directed mutagenesis of single-stranded M13 DNA, using standard molecular biology methodology. Digestion of this mutagenized clone

DNA with BclI and HindIII yielded a 370 bp fragment containing the entire $sPLA_2$ coding region with the exception of the first nine amino acid residues of the mature protein. These nine residues, along with a cleavable fusion site (Trp) and an EcoRI site were replaced with the two oligonucleotide linkers shown in Figure 8. The expression construct p86-1A was obtained by ligating the 370 bp BclI-HindIII fragment along with the two oligonucleotides into expression vector pHNF86 which had been previously cut with EcoRI and HindIII. The pHNF86 vector consists of a pBR322 backbone, the E. coli tryptophan promoter, a ribosomal binding site, sequences encoding a portion of the amino terminal portion of the E. coli β-galactosidase gene followed by six Thr residues, an EcoRI and HindIII site, and two strong E. coli transcription termination signals. See, e.g., Sung et al (1986) Proc Natl Acad Sci USA 83:561-565.

The resulting expression vector containing the $sPLA_2$ construct was then used to transform E. coli strain W3110 (ATCC accession no. 27325) for expression. After inoculation of a culture of transformed cells of a suitable population density, expression was induced by the addition of 3-β-indoleacrylic acid into the media. After 9 hours of growth in induction media, inclusion bodies were observed in about 90% of the cells. After cell disruption, the inclusion bodies were then pelleted and boiled 5 min in gel loading buffer containing 50 mM β-mercaptoethanol. By comparison with gels of similar extracts from uninduced and control cultures, a prominent 15 Kd band was observed in the induced cultures transformed with the expression construct. This band was highly enriched in gels from extracts of purified

inclusion bodies, allowing large-scale isolation of this fusion protein from preparative SDS-polyacrylamide gels. Fusion protein prepared in this manner was injected into rabbits, rats and mice for the production of antibodies.

Purified fusion protein fractions may be activated by the S-sulfonation procedure as described by DeHaas et al. (1987), supra. After activation, the fusion protein can be cleaved at the Trp residue to release the mature human PLA_2. Alternatively, the two steps in reverse order may give greater yields of active protein. See, e.g., Lishchwe & Ochs (1982) Anal Biochim 127:453-457. A further conventional purification step can then be used to separate the $sPLA_2$ from the β-gal leader.

B. Vaccinia Virus

Recombinant $sPLA_2$ polypeptides can also be provided in mammalian cells using a vaccinia virus expression vector. Such expression vectors are well known in the art. See, e.g., PCT Pub. No. WO 86/07593 (CBI:PB8).

For example, the vaccinia expression vector pSC-11, described in Chakrabarti (1985) Mol Cell Biol 5:3403, was employed according to the following protocol. An $sPLA_2$ coding fragment was prepared as described above, except that base 127 was changed from A to G via oligonucleotide-directed mutagenesis to produce a SacI site 5 bp upstream from the initiation ATG codon. The coding region is thus contained on a 469 bp SacI-HindIII fragment. This fragment was then blunt-end ligated into vaccinia vector pSC-11, previously cut with SmaI. The resulting DNA was recovered and used to transfect

vaccinia-infected monolayers of cultured mammalian CV-1 cells using standard procedures. The resulting plaques were purified through several rounds of infection.

As shown in Figure 9, assays of PLA_2 activity present in both cells and media showed significant accumulation of PLA_2 in the media with time. Large quantities of media prepared in a similar manner can be obtained, and the active recombinant PLA_2 enzyme expressed by these cells can thus be obtained by standard purification procedures. In addition, the infectious virus purified from these cells was used to vaccinate rabbits and mice for the production of antibodies recognizing the active enzyme. When a significant titer is achieved in mice, monoclonal antibodies blocking the enzyme activity can be identified by screening hybridoma clones for the ability to block activity in the PLA_2 assays described above.

V. Inhibitory Antibodies to Synovial PLA2

Two peptides corresponding to segments of the human synovial PLA2 sequence: (i) 67-85 (GTKFLSYKFSNSGSRITC) and (ii) 109-132 (NKTTYNKKYQYYSNKHSRGSTPRC) were synthesized, coupled to ovalbumin with glutaraldehyde and used to separately immunize rabbits.

Antisera were obtained to both peptide conjugates with titres of 1:40,000 as determined by an Elisa assay. IgG was purified from the antisera and control sera by the method of McKinney and Parkinson (J Immun Meth 96:271-278, (1987)) and used in in vitro activity assays at a concentration of 10 mg/ml.

The source of synovial PLA2 activity for the assay was a partially-purified preparation of Chinese hamster ovary cell conditioned medium from cells transfected with the synovial PLA2 sequence under the transcriptional control of the human metallothionein promoter. Partial purification was achieved by ion exchange chromatography (MonoQ column, 0-2 M NaCl gradient in 50 mM Tris-HCl, pH 8.0), dialysis and lyophilization.

The in vitro activity assay was performed as described above, the modification that the enzyme and IgG were preincubated in assay buffer for 1 hour at 37°C prior to the addition of substrate.

Preincubation with antibodies to each peptide results in approximately 50% inhibition of activity relative to control IgG. The results are shown in Table IV:

Table IV

Inhibition of PLA_2 by Monoclonal Antibodies

Sample	% Hydrolysis
control IgG	43%
pep1IgG	25%
pep2IgG	17%

While the present invention has been illustrated above by certain specific embodiments, it is not intended that these specific examples should limit the scope of the invention as described in the appended claims.

CLAIMS

1. A composition comprising double-stranded DNA constructs containing a heterologous region comprising a coding sequence for a mammalian synovial phospholipase A_2 ($sPLA_2$), said composition substantially free of constructs not containing said heterologous region.

2. The composition of claim 1 wherein said coding sequence encodes the amino acid sequence of mature $sPLA_2$.

3. The composition of claim 1 wherein said coding sequence encodes the amino acid sequence of the preenzyme.

4. The composition of claim 1 wherein said mammal is human.

5. The composition of claim 1 wherein said $sPLA_2$ is soluble.

6. The composition of claim 1 wherein said $sPLA_2$ is a mutein and its amino acid corresponding to His_{48} in the native $sPLA_2$ sequence is not His.

7. The composition of claim 6 wherein said amino acid corresponding to His_{48} is Glu or Asp.

8. The composition of claim 1 wherein said DNA constructs further comprise a replicon.

9. The composition of claim 8 wherein said replicon is a bacterial plasmid.

10. The composition of claim 8 wherein said replicon is a yeast plasmid.

11. The composition of claim 8 wherein said replicon is a bacteriophage.

12. The composition of claim 8 wherein said replicon is a chromosome.

13. A method of producing a recombinant mammalian synovial phospholipase A_2 ($sPLA_2$) comprising:

providing a population of transformed cells containing a replicon functional in said cells, said replicon comprising a coding sequence under the control of a promoter functional in said cells, said coding sequence encoding a mammalian $sPLA_2$, said population being substantially free of other cells;

growing said population under conditions whereby said mammalian $sPLA_2$ is expressed; and

recovering said mammalian $sPLA_2$.

14. The method of claim 13 wherein said cells are bacterial cells.

15. The method of claim 13 wherein said cells are yeast cells.

16. The method of claim 13 wherein said cells are mammalian cells.

17. The method of claim 14 wherein said replicon is a plasmid.

18. The method of claim 15 wherein said replicon is a plasmid.

19. The method of claim 16 wherein said replicon is a chromosome.

20. The method of claim 13 wherein said mammal is human.

21. A composition comprising mammalian synovial phospholipase A_2 ($sPLA_2$) substantially free of contaminating proteins.

22. A composition according to claim 21 wherein said $sPLA_2$ is $sPLA_2$ type A.

23. A composition according to claim 21 wherein said $sPLA_2$ is $sPLA_2$ type B.

24. A composition according to claim 21 wherein said $sPLA_2$ is $sPLA_2$ type C.

25. A composition according to claim 21 wherein said $sPLA_2$ is a mutein and the amino acid corresponding to His_{48} in the native $sPLA_2$ is not His.

26. A composition according to claim 25 wherein said amino acid corresponding to His_{48} is Glu or Asp.

27. A composition comprising antibodies recognizing an epitope unique to a mammalian synovial phospholipase A_2.

28. A therapeutic method comprising administering to a mammal suffering from an inflammatory disorder an effective amount of an anti-mammalian synovial phospholipase A_2 antibody composition.

29. A method comprising providing a synovial fluid sample from a mammal, and measuring the amount of mammalian synovial phospholipase A_2 ($sPLA_2$) type A and type B in said sample in a quantitative assay.

30. A method according to claim 29 wherein said quantitative assay is an immunoassay.

31. In a vaccine composition comprising an antigen, a pharmaceutically acceptable parenteral vehicle, and at least one adjuvant, the improvement comprising using a mammalian synovial phospholipase A_2 ($sPLA_2$) as an adjuvant.

FIG. 1

COMPARISON OF PLA$_2$ AMINO ACID SEQUENCES

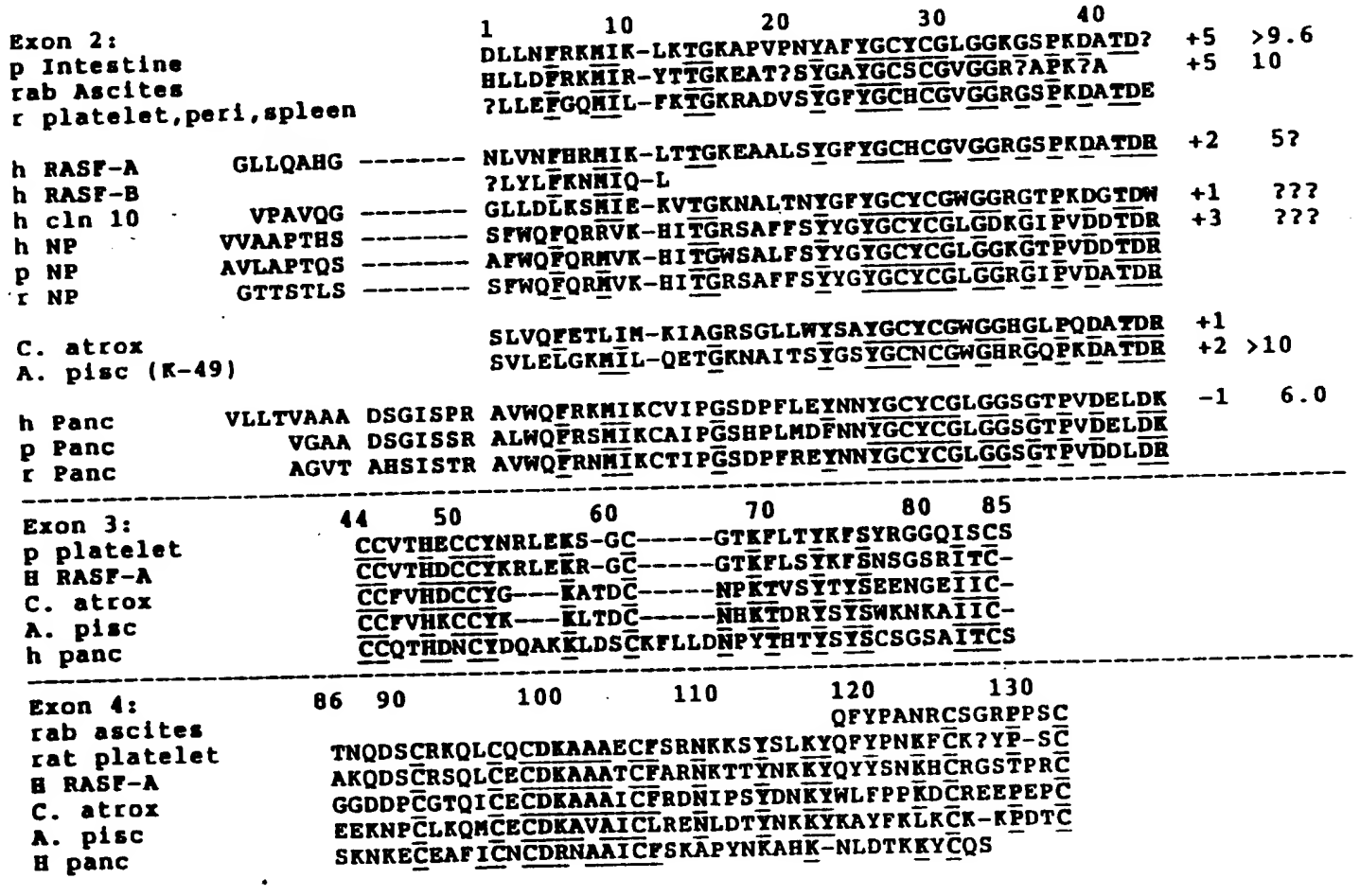


FIG. 2

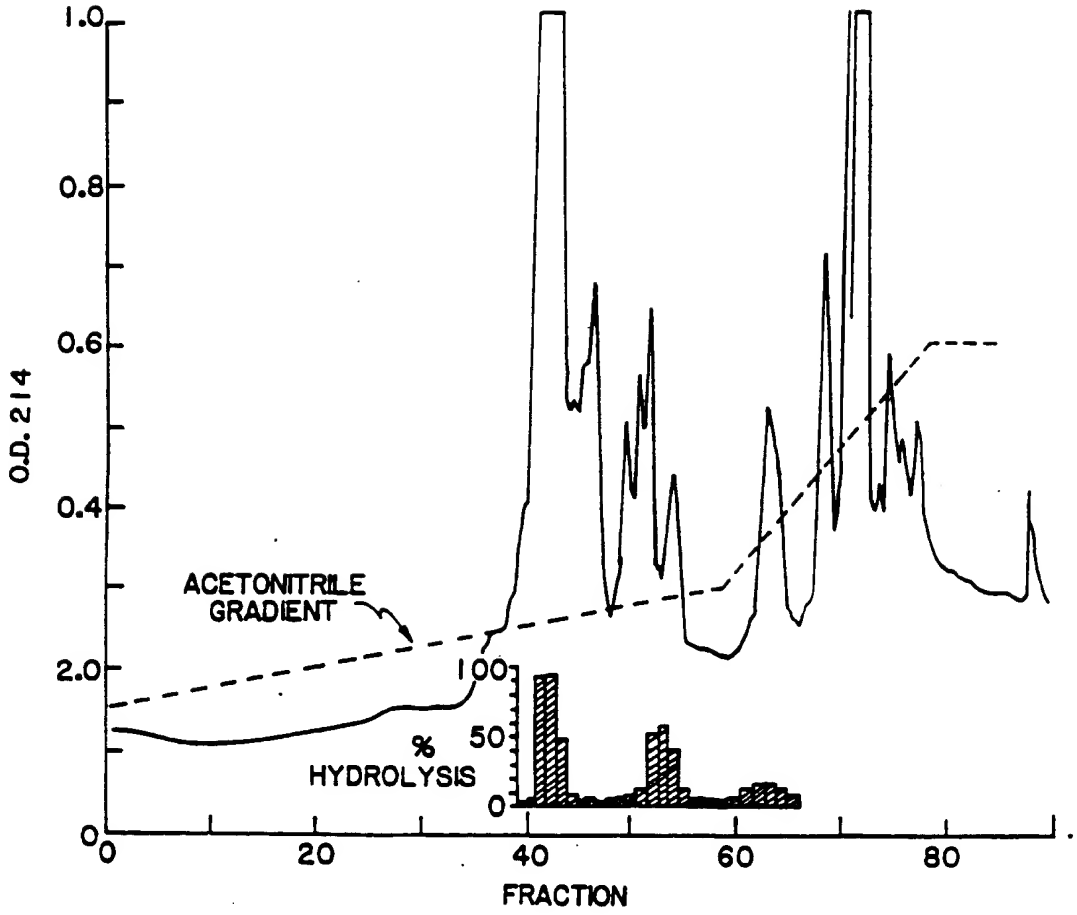

1.0
0.8
0.6
0.4
2.0
0
O.D. 214
ACETONITRILE
GRADIENT
100
50
0
%
HYDROLYSIS
0
20
40
60
80
FRACTION

FIG. 3

Screening oligos based upon RASF peak A Amino Acid Sequence:

```
 1                                        16        20                                32
AsnLeuValAsnPheHisArgMetIleLysLeuThrThrGlyLysGluAlaAlaLeuSerTyrGluPheTyrGlyCysHisCysGlyValGly   RASF-A
AATTTGGTGAATTTCCACAGAATGATCAAGTTGACGACAGGAAAGGAAGCCGCACTCAGTTATGGCTTCTACGGCTGCCACTGTGGCGTGGGT  Clone 6

                        Oligo 2779 ->  3'-TTTCTTCGGCGGGATAGGATGCTTAAGATGCCGACGGTGACGCCGCATCC-5'
  3'-CATTTGAAGGTGTCTTACTAGTTTGATTGGTGGCCGTTTCTTCGGCGGGA-5'  <- Oligo 2780
```

FIG. 5

```
   5                                                    24      position #
  PheHisArgMetIleLysLeuThrThrGlyLysGluAlaAlaLeuSerTyrGlyPheTyr      AA sequence
5'-TTCCACAGAATGATCAAGTTGACGACAGGAAAGGAAGCCGCACTCAGTTATGGCTTCTAC-3'  gene seq.

3'-AAGGTGTCTTACTAGTTCAACTGCTGTCCTTTCCTTCGGCGTGAGTCAATACCGAAGATG-5'  Oligo 2905
```

FIG. 4-1

Human Genomic Clone 6 (Alu 404)
RASF Peak A Gene

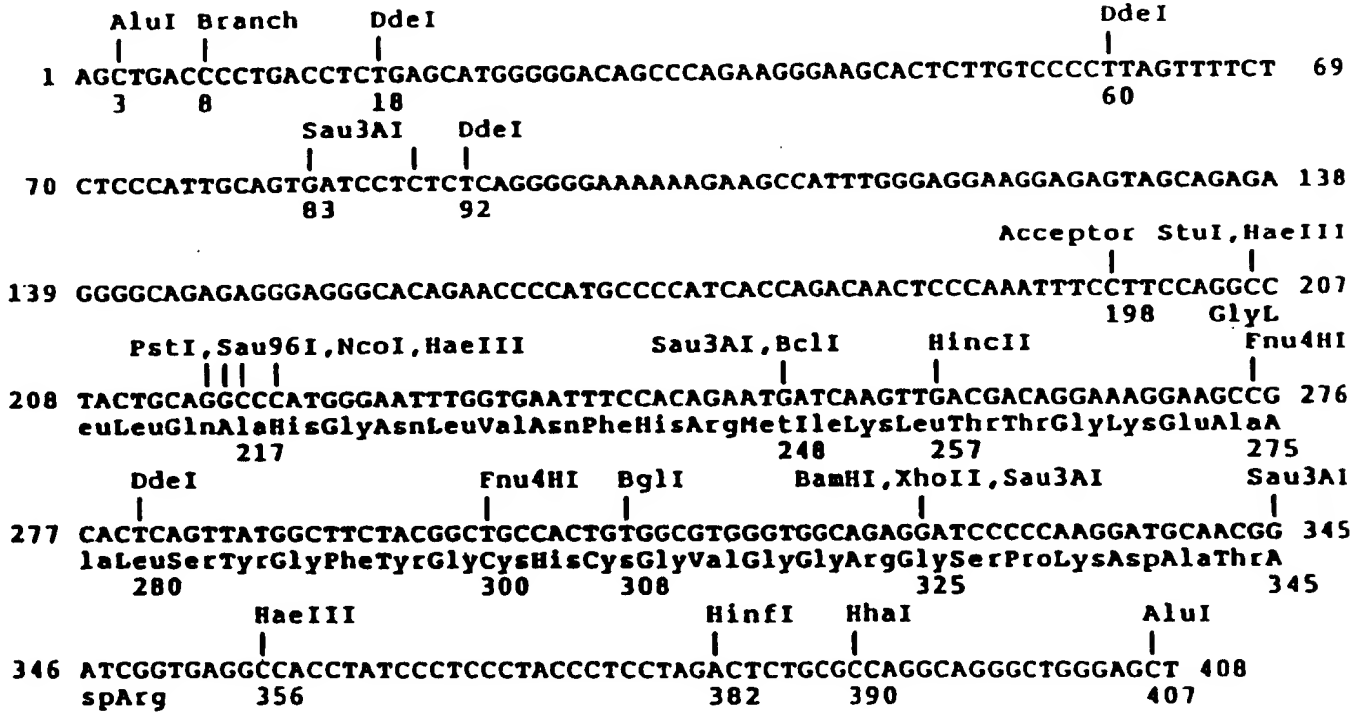


FIG. 4-2

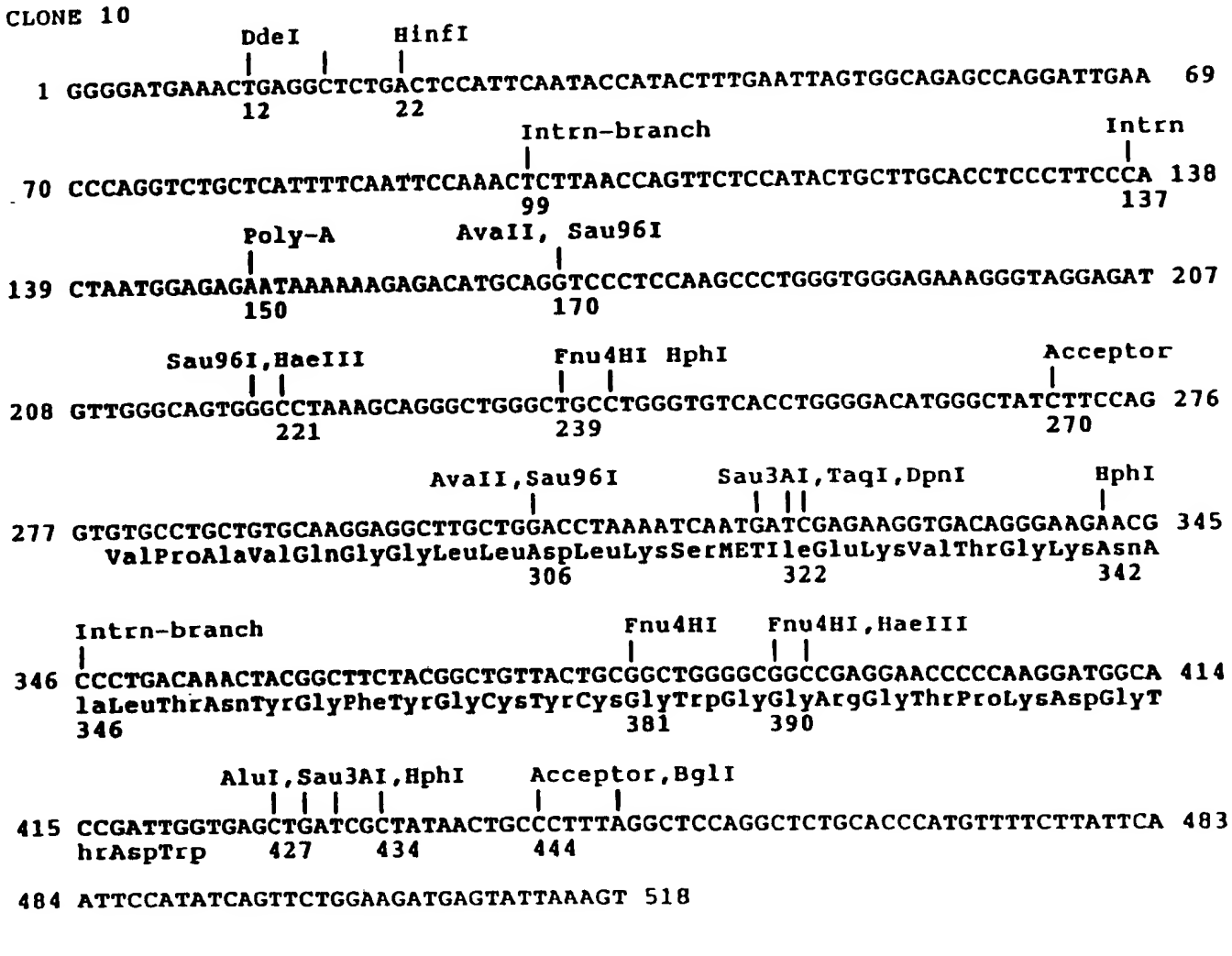


FIG. 6

```
                                        <1>
   1  GAATTCCCAACTCTGGAGTCCTCTGAGAGAGCCACCAAGGAGGAGCAGGGGAGCGACGGC   60
   1                                                                  60
  61  CGGGGCAGAAGTTGAGACCACCCAGCAGAGGAGCTAGGCCAGTCCATCTGCATTTGTCAC  120
  61                                                          <2>    120
 121  CCAAGAACTCTTACCATGAAGACCCTCCTACTGTTGGCAGTGATCATGATCTTTGGCCTA  180
 121                  MetLysThrLeuLeuLeuLeuAlaValIleMetIlePheGlyLeu  180
 121                  -20                                            180
 181  CTGCAGGCCCATGGGAATTTGGTGAATTTCCACAGAATGATCAAGTTGACGACAGGAAAG  240
 181  LeuGlnAlaHisGlyAsnLeuValAsnPheHisArgMetIleLysLeuThrThrGlyLys  240
 181                  +1        T                                    240
 241  GAAGCCGCACTCAGTTATGGCTTCTACGGCTGCCACTGTGGCGTGGGTGGCAGAGGATCC  300
 241  GluAlaAlaLeuSerTyrGlyPheTyrGlyCysHisCysGlyValGlyGlyArgGlySer  300
 241                       <3>                                       300
 301  CCCAAGGATGCAACGGATCGCTGCTGTGTCACTCATGACTGTTGCTACAAACGTCTGGAG  360
 301  ProLysAspAlaThrAspArgCysCysValThrHisAspCysCysTyrLysArgLeuGlu  360
 301                                                                 360
 361  AAACGTGGATGTGGCACCAAATTTCTGAGCTACAAGTTTAGCAACTCGGGGAGCAGAATC  420
 361  LysArgGlyCysGlyThrLysPheLeuSerTyrLysPheSerAsnSerGlySerArgIle  420
 361       <4>                                                       420
 421  ACCTGTGCAAAACAGGACTCCTGCAGAAGTCAACTGTGTGAGTGTGATAAGGCTGCTGCC  480
 421  ThrCysAlaLysGlnAspSerCysArgSerGlnLeuCysGluCysAspLysAlaAlaAla  480
 421                                                                 480
 481  ACCTGTTTTGCTAGAAACAAGACGACCTACAATAAAAAGTACCAGTACTATTCCAATAAA  540
 481  ThrCysPheAlaArgAsnLysThrThrTyrAsnLysLysTyrGlnTyrTyrSerAsnLys  540
 481                                                                 540
 541  CACTGCAGAGGGAGCACCCCTCGTTGCTGAGTCCCCTCTTCCCTGGAAACCTTCCACCCA  600
 541  HisCysArgGlySerThrProArgCys                                    600
 541                                                                 600
 601  GTGCTGAATTTCCCTCTCTCATACCCTCCCTCCCTACCCTAACCAAGTTCCTTGGCCATG  660
 601                                                                 660
 661  CAGAAAGCATCCCTCACCCATCCTAGAGGCCAGGCAGGAGCCCTTCTATACCCACCCAGA  720
 661                                                                 720
 721  ATGAGACATCCAGCAGATTTCCAGCCTTCTACTGCTCTCCTCCACCTCAACTCCGTGCTT  780
 721                                                                 780
 781  AACCAAAGAAGCTGTACTCCGGGGGGTCTCTTCTGAATAAAGCAATTAGCAAATCAAAAA  840
 781                                                                 840
 841  AAAAAAAGGAATTC  854
 841                  854
```

KEY:
<1> intron positions
+1 amino acid positions

sPLA$_2$ Gene Sequence (Exons 1-5)

```
   1 GCAAGGGGCTCTAAGAATTGTAAGGGAACAGATGGATGTTCACAAGCACCACAGCCCTGGCCACATGACT 70
  71 TTTTAGGACTGGTATCGCAGAGTGTTTACTTAAGGCGGTGGAAGCTAAATTCTTAGCATGTGCTGGAGAG 140
 141 CATGAAAAAGATATTTACTTTATGAATTAAAGCTGGAGTCAGTGTCAGCCCGAAGGTGAAGGAAAAAGAG 210
 211 CAACAGATCCAGGGAGCATTCACCTGCCCTGTCTCCAAACAGGTGAGGATGGGGAATAAAGTGAAGGGCA 280
 281 GTGCTTTGGTGGGAACTTCAAGGATACGCTCTGGCTTTTTCCAGGTTTAGAAGCTCATATGAGACAGGGG 350
 351 TGGAGGAAAAGAAGAAAGAAGAATAAGAAGAGAAAGTTGAGGCCCTGGCCCAAGTTAGTGGGAAGGAAAT 420
 421 CCACCCCATTAAACTCTCTCCCTGTGGACTTGGGTCACACGTGAGGCCTGCACAGTGCTGGAACATGGTA 490
 491 GAGGCCCAGGACATACTTCCTGTGAATGAATGATTGAGCGGCTGAATGAATGAGTACCGCTAAAAGCCCT 560
 561 CTTTTCTATTCCCAAATGCCACATTGAGCAGAAGGGAGCAGAGATCCTTGCTCAGCAATTGGTAGTCCCA 630
 631 TTTGGGTGTGCAAATGAGTCCACAGCCTGCAACAGCAGACAGTCTCTGCCCCCCTTAGAGGCGATTGCAG 700
 701 GGAGGTGGCTGACCGTTGATCACACCCAGAGGCTGGTTATGGGAATTTACTCCATGGAAAGACTCGGCAA 770
 771 AACTGCCTGAATGTGTTTTGGCATCAGGCTACTGACACGTAAGGGTTTCCCAATCCTCAACTCTGTCCTG 840
 841 GCCAGGCTGATGAGGGGAAGGAAAGGGATTACCTAGGGGTATGGGCGACCAATCCTGAGTCCACCAACTG 910
 911 ACCACGCCCATCCCCAGCCTTGTGCCTCACCTACCCCCAACCTCCAGAGGGAGCAGCTATTTAAGGGGAG 980
1> 981 CAGGAGTGCAGAACAAACAAGACGGCCTGGGGATACAACTCTGGAGTCCTCTGAGAGGTAAAGAGCCAGC 1050
1051 GAAGCTGATGTCCTGTCAAGAGCAGAATTC 1080

   1 GAATTCCTGCTCATTCGCTGCCTTTGAGAGTGGCTGTGTTGTGCATGCATGTGCATGATTTGATATGTAT 70
  71 GAGAGGGTGTGTGTGCATGAGTGTGTTGAGTGAGTATGTGAGTGTAGTGTAAGAGAGGATGTTGGCACTA 140
 141 TCAGGTAATTACGAGAGTGTGTGTATGTGGGCATAGGTGTGTTAACATGTATGTGTTTGGGAACTTGTGT 210
 211 ATGTGGAAGGGGTTAGAAGGCCTAGAAGAGAGAGGTTGATGCTTTCATTCTGGAGGAAAATACTGAGGCC 280
 281 GAGCCTCCATGGGTGCCTTGGAGACTCCAAGCCTTGAATCCAGTGTGGGGATATGCAAGCTATGTCTAGC 350
 351 GAGGGACACATCCTCTGACCTCAGGAACCTCCCAGGTAGTTGGGAGGAACCTGGTTCCAACCTCCCAAGA 420
 421 ACTCTCAGTCTGATGACGGTACAGGGGAGGTCTCATTAGTGTATCATGGGGTTCTCCACAGGTCTGAGGG 490
 491 CCTGATGTGTGTGAAACCATTCTGCAGAGCTGGGAACGGGTCAGGAGGTGGTTGTGTGTGTGTGTGTG 560
 561 TGTGTGTGCATTGCTGGAGGGCACTCCTTGTGTGCTCTGAGTGTGACAGAGGAAGTCACCCTGGACTTAG 630
2> 631 GTTGGATGGGAGAGCCATGTCTGTGTGTCTCAGAGCCACCAAGGAGGAGCAGGGGAGCGACGGCCGGGGC 700
```

FIG. 7 – 1

```
2>  701 AGAAGTTGAGACCACCCAGCAGAGGAGCTAGGCCAGTCCATCTGCATTTGTCACCCAAGAACTCTTACCA 770
                                                                          M

2>  771 TGAAGACCCTCCTACTGTTGGCAGTGATCATGATCTTTGGTAAGAGCTGACCCTGACCTCTGAGCATGGG 840
          etLysThrLeuLeuLeuLeuAlaValIleMetIlePheG

    841 GGACAGCCCCAGAAGGGAAGCACTCTTGTCCCTTAGTTTTCTCTCCCATTGCAGTGATCCTCTCTCAGGG 910

    911 GGAAAAAAGAAGCCATTTGGGAGGAAGGAGAGTAGCAGAGAGGGGCAGAGAGGGAGGGCACAGAACCCCA 980

3>  981 TGCCCCATCACCAGACAACTCCCAAATTTCCTTCCAGGCCTACTGCAGGCCCATGGGAATTTGGTGAATT 1050
                                                lyLeuLeuGlnAlaHisGlyAsnLeuValAsnP

3> 1051 TCCACAGAATGATCAAGTTGACGACAGGAAAGGAAGCCGCACTCAGTTATGGCTTCTACGGCTGCCACTG 1120
          heHisArgMetIleLysLeuThrThrGlyLysGluAlaAlaLeuSerTyrGlyPheTyrGlyCysHisCy

3> 1121 TGGCGTGGGTGGCAGAGGATCCCCCAAGGATGCAACGGATCGGTGAGGCCACCTATCCCTCCCTACCCTC 1190
          -GlyValGlyGlyArgGlySerProLysAspAlaThrAspAr

    1191 CTAGACTCTGGCCCAGGCAGGGCTGGGAGCTGCAAAGACAGTGCCGGTTCCTGATGGGCGCAGAGGTCTC 1260

    1261 AGGATGGCCTGGCTGGAAAAGCAGCCGGCATGTTGGAACTTCTGCTCTAGACTGTTGCAAAGTCACTGGG 1330

    1331 TCTCTGCCCAGGGTCCAAGGGGGTGAGACCACAGGCACCAGGCCTCCTGGAGCTGTGGGACAAGAGCCCC 1400

4>  1401 AACAGGTGTCTCCTCACAGCTGCTGTGTCACTCATGACTGTTGCTACAAACGTCTGGAGAAACGTGGATG 1470
                                gCysCysValThrHisAspCysCysTyrLysArgLeuGluLysArgGlyCy

4>  1471 TGGCACCAAATTTCTGAGCTACAAGTTTAGCAACTCGGGGAGCAGAATCACCTGTGGTAAGAGTCCTACC 1540
          sGlyThrLysPheLeuSerTyrLysPheSerAsnSerGlySerArgIleThrCysA

    1541 TCACCATCGAGTGGCCCTCATTTGTTTAGACAGTGCTGGGACTGTGCTGGGCACCAAAGATAGACACAGA 1610

    1611 GGGACACAGTTCCTGCTTCAGGAAGCTCACGGTTGAGTGGGAAGCCAGGAAAGTGAAAATCCAATGTAGT 1680

    1681 AAAGACTCCAGTGGGAAGTAAACAAACAGATAAGGCATTAACACAGCCTGAGGCTTGAGGAAGGCTCCTG 1750

    1751 GAAGGGGTGACCCCTAAGCTGAGTCTGAAAGGCTGTGCAGAGAGTCAGGGAAGAGGAGGGAGCATTCCCA 1820

    1821 GAAGAGGACACAGCATGGTCAAAGGCACTAAAGGGCACTGTAAGCCATTCTGTACTGCCCAGCAGAAACA 1890

    1891 TGAGGAAGAGGAGCAGTGCTGAGCCATGATGCTGGAGACATAGGAAGGAGCTAGGTCAATCCGCCCTCCA 1960

    1961 CTCCGGGCTGTATTTAGGTTTTGCCCTAAAGCAATAGGATGCTATTAAGCAAAGGAGCTACAGGGTCAGA 2030

    2031 TTTGCATTTTAGATGACTCACTGTGGGGACAGGGTCGATGGAGACAAGTGGAAGGGGGCAGAGAAAGCTA 2100

    2101 TTGCCATCATGCAGGCAAGAGGGAGTAACATCTTGACATAAAACAATGGAGGTCAGGATGGGAAAGGTGG 2170

    2171 AGAAAAAATCAAGATGCATTTGAGATGGAATGCAGCTGAACTGGTGACTGAGTTGGGAGGGATGGGGAGA 2240

    2241 GGGAGTTGTTGGATGGATATGTGGCTGCATGGATGGCACAACTGTGATAAAGACCATGGGAGCAGGTCAT 2310

    2311 GGTGGAGGGTGGGGAGGAGCAGTTCTATTTCCAGCATGTTGAGTTTAGGGGCCTCCAGCACCCAGGGAGG 2380
```

FIG. 7-2

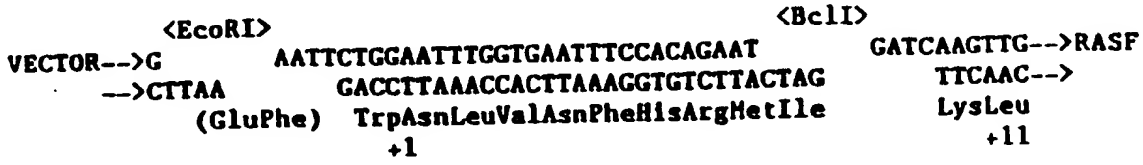
```
2381 GGTCCAGCAGGCAGCTGTCTATACAAATGCAGCTCAGGGGAGAATTCAGGACTGGGACACAGATTCAGAA 2450

2451 GCCAGCAGCAGAGACCTGAGAGGTGGGTGTGATCACTCATTTGCTGTTTAAAGGCCCAGAAAGGAGACAG 2520

2521 AGAAGGGATGGACAGAGAGGGAGAAGGGGAACTGAGCGAGAAGGTCAAGGAGTCAGTAAGGAAATGGTTA 2590

2591 GCAAGGGCCAAGTGAACAGGGAGTCCTCCATGAAAAGGGCCAACAAGGCTCCCCTGGATGTTGAGGCAGA 2660

2661 AACGCATGAGGGACTCAGGGAAGCTGTTTCCATGGAGTCGGGAGGGCAAAGCCAGATTAGACCAGGTGGG 2730

2731 GGCTGATGGGAAGGCAAATAAAGACAGGAGGCAAAGACAACATTCTGGAGAAAGTTTGGCCTGAAGGGAG 2800

2801 GAGAGTGGTGGCACTGGAAGGCTTTGCTTGGTGTCCCCAGACAGCTGACTCATGAGTGGGATTTGGAAAA 2870

2871 AGCGTGGACTCCTGCCCATGGCCTGAGTCCTTTAAGATCAGAAATTATGTCTCCCATCATGGCCTCTCCA 2940

2941 TAGAGGCATGTATCTTCAGCAGGCGTTAGGTCACAAGCCACATGATGCCAAGCTGACAGTGGCTTGCATA 3010

3011 ATGGGGATATGTGACTGTCGCATAACTAGAATTCTGGAAGAGTGCAGTGCCAGGCTTGGGGCAGCTTTCC 3080

3081 AGCCATGTCATTAAGAATCCAGCCTTCTCCTGGCCTTCAGCTATGCCACGTGGCCAGTGTCTACACCTGG 3150

3151 GATGTCAAGAGACAGGCTGCAGGTCCACCCTCCTGGCCTCATACTATGGAAGAGGCTTTCCTTTTGGGCA 3220

3221 TCTCTCTTTTTGGAGGGAGGAAATAGATCGTTCCCAGAAGCCCCCAGCAGACTTCCCCTTGTTGCTCATT 3290

3291 GGTTGGAACAAGGTTACATGATACACAAAGACCAATCACTGCAAAGGAAAAAGGGATGACCCTGCCTGGC 3360

3361 TTACACCAATCACAATCTATTCCCAGACCCCCCGAGGCTAGGGCTTTGCCTCCTGGACACATCTGTTAGC 3430

3431 AAGAGGAAGAGATTATGGCTGTTAGGAAGGCCTTTGAGAAAGTATCCCAGTGCCTGGCTGTGTCTCCACC 3500

3501 AGGCTGGAGGCCAGCATCCCAAGGGCAAGAATTCTGTCTCCCCATTGGTCAGAAATATCTGGAGCGCAGG 3570

3571 TGTTTGTCTCCAACTAGGAGCTTCTGGAGGACAGGGCTGTGTCTTCTACCCCAGGGTTCCACAAGAAGCC 3640

5>3641 ACTGAATATTAATAAAGTCCCATCTTGTGTTTATTTCTTATGATTTCAAAACAGGACTCCTGCAGAAGT 3710
                                                    laLysGlnAspSerCysArgSer

5>3711 CAACTGTGTGAGTGTGATAAGGCTGCTGCCACCTGTTTTGCTAGAAACAAGACGACCTACAATAAAAAGT 3780
       GlnLeuCysGluCysAspLysAlaAlaAlaThrCysPheAlaArgAsnLysThrThrTyrAsnLysLysT

5>3781 ACCAGTACTATTCCAATAAACACTGCAGAGGGAGCACCCCTCGTTGCTGA...  3830
       yrGlnTyrTyrSerAsnLysHisCysArgGlySerThrProArgCys***
```

FIG. 7-3

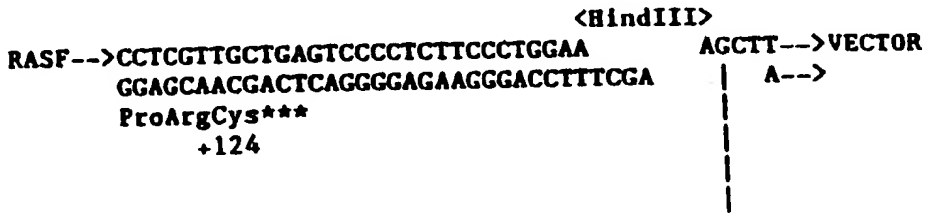
FIG. 8

RASF Procaryotic expression vector Construction

N-terminal Hookup:



C-terminal hookup:



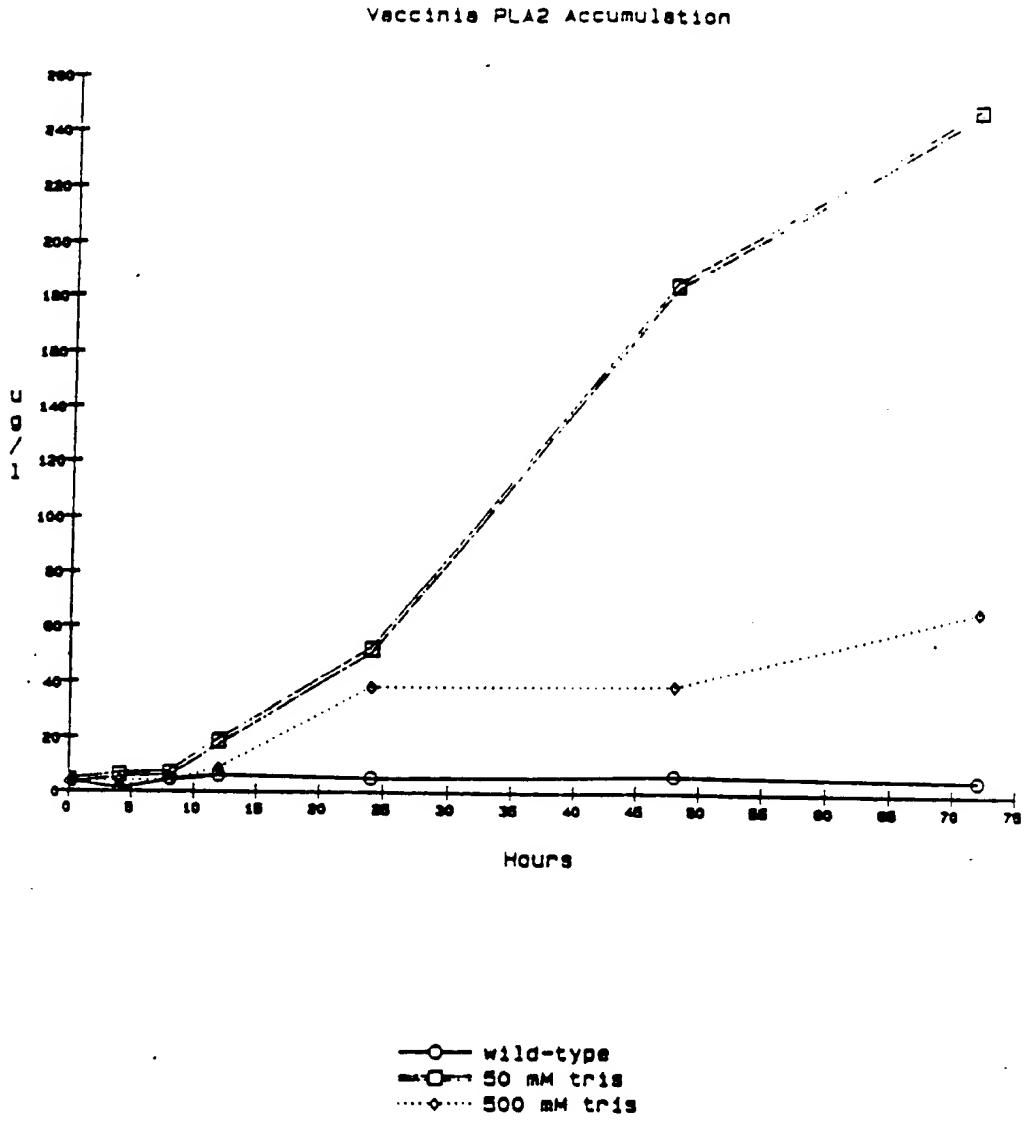

Vaccinia PLA2 Accumulation
ug/l
Hours
wild-type
50 mM tris
500 mM tris

FIGURE 9

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02896

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4) A61k 9/20;C07H 15/12;C12N 15/00; C12Q 1/00
US. CL 424/94;536/27;435/198;435/172.3;435/320;435/7;

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/7, 172.3, 198, 320 424/94; 536/27 935/14

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

CHEMICAL ABSTRACTS DATA BASE (CAS)1967-1988; BIOLOGICAL DATA BASE (BIOSIS) 1967-1988. KEYWORDS: MAMMALIAN, PHOSPHOLIPASE, PLASMID, VECTOR, RECOMBINANT, GENE

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	NUCLEIC ACIDS RESEARCH (OXFORD, UK) VOLUME 15, issued 11 May 1987 (GEUS ET AL) "Expression of porcine phospholipase A_2. Generation of active enzyme by sequence-specific cleavage of a hybrid protein from Escherichia coli", see pages 3743-59.	1-31
Y	BIOCHEMISTRY (WASHINGTON, D.C., USA) issued 24 March 1987 (HAN ET AL) "Isolation of full-length putative rat lysophospholipase cDNA using improved methods for mRNA isolation and cDNA cloning", see pages 1617-25.	1-31

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21 November 1988	05 JAN 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Thomas D. Mays

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	DNA (NEW YORK, USA) issued December 1986 (SHEILHAMER ET AL) "Pancreatic phospholipase A_2: Isolation of the human gene and cDNA's from porcine pancreas and human lung", see pages 519-27.	1-31
Y	JOURNAL OF BIOCHEMISTRY (TOKYO, JAPAN) issued February 1986 (HARUKI ET AL) "Interaction of monodispersed and micellar phospholipids with an Agkistrodon halys blomhoffii phospholiphase A_2, in which the alpha-amino group had been modified to an alpha-keto group", see pages 99-109.	1-31